A β-Tubulin Leucine Cluster Involved in Microtubule Assembly and Paclitaxel Resistance*§

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Analysis of β-tubulin alleles from nine paclitaxel-resistant Chinese hamster ovary cell lines revealed an unexpected cluster of mutations affecting Leu-215, Leu-217, and Leu-228. Six of the mutant alleles encode a His, Arg, or Phe substitution at Leu-215; another mutant allele has an Arg substitution at Leu-217; and the final two mutant alleles have substitutions of His or Phe at Leu-228. Using plasmids that allow tetracycline regulated expression, the L215H, L217R, and L228F mutations were introduced into a hemagglutinin antigen-tagged β-tubulin cDNA and transfected into wild-type Chinese hamster ovary cells. In all three cases, low to moderate expression of the transfected mutant gene conferred paclitaxel resistance. Higher levels of expression caused disruption of microtubule assembly, cell cycle arrest at mitosis, and failure to proliferate. Consistent with reduced microtubule stability, cells expressing mutant hemagglutinin β-tubulin had fewer acetylated microtubules than nonexpressing cells in the same population. These data, together with previous studies showing that the paclitaxel-resistant mutant cell lines have less stable microtubules, indicate that the leucine cluster represents an important structural motif for microtubule assembly.

Paclitaxel is the prototype for a novel class of agents that inhibit cells in mitosis by promoting and stabilizing microtubule assembly. Early studies with this compound demonstrated that it binds to microtubules in a 1:1 stoichiometry with tubulin heterodimers (1) and inhibits microtubule disassembly. It is also able to induce microtubule assembly both in vitro and in vivo and induces microtubule bundle formation in treated cells (2, 3). Recent interest in this and related compounds has been fueled by clinical studies demonstrating remarkable activity of paclitaxel against a number of malignant diseases (reviewed in Ref. 11). Based on mutant properties and drug resistance mechanisms, we have now sequenced β-tubulin alleles and find that the mutations at a site that is likely to be involved in lateral or longitudinal interactions during microtubule assembly.

The mechanisms by which tumor cells acquire resistance to paclitaxel are not fully understood. Cell culture studies have shown that paclitaxel is a substrate for the multidrug resistance pump (gp170), and cells selected for high levels of resistance to the drug have increased gp170 (reviewed in Ref. 5). Nevertheless, it has yet to be demonstrated that this mechanism is significant in paclitaxel refractory tumors. Indeed, the remarkable efficacy of paclitaxel in early clinical studies of patients who were pretreated with Adriamycin, a well known substrate for gp170, argues that the multidrug resistance (mdr) phenotype may not be as clinically prevalent as had initially been anticipated (4).

Additional mechanisms of resistance to paclitaxel have been reported. For example, several laboratories have provided evidence that changes in the expression of specific β-tubulin genes are associated with paclitaxel resistance in cultured tumor cell lines (6–9). More recently, a report describing mutations in β-tubulin that make the protein unresponsive to paclitaxel has appeared (10). To date, however, there is little evidence that any of the mechanisms described in cell culture cause paclitaxel resistance in human tumors.

Our own studies have described a resistance mechanism mediated by tubulin alterations that affect microtubule assembly (reviewed in Ref. 11). Based on mutant properties and drug cross-resistance patterns, we proposed that these changes in microtubule assembly could compensate for the presence of the drug (12); and we were later able to directly demonstrate that paclitaxel-resistant Chinese hamster ovary (CHO) cells have diminished microtubule assembly compared with wild-type controls (13). Thus, isolation of paclitaxel-resistant mutants provides an opportunity to study mutations that not only give information about the mechanisms of drug action and resistance, but also give structural information about regions of tubulin that are involved in assembly. We have now sequenced nine mutant β-tubulin alleles and find that the mutations cluster at a site that is likely to be involved in lateral or longitudinal interactions during microtubule assembly.

MATERIALS AND METHODS

Isolation, Maintenance, and Labeling of Cell Lines—Paclitaxel-resistant CHO mutants used in this study and conditions for their growth in α modification of minimum essential medium (α-MEM, Life Technologies, Inc.) have been described previously (14–16). Metabolic labeling was for 1 h in methionine-free MEM (ICN Biomedicals Inc., Costa Mesa, CA) containing 30 μCi/ml Tran35S-label (1,000 Ci/mmol; ICN Biomedicals).

Sequencing Mutant β-Tubulin—To analyze mutant alleles, we first

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The abbreviations used are: gp170, multidrug resistance pump; α-MEM, α modification of minimum essential medium; CHO, Chinese hamster ovary; mdr, multidrug resistance; tTA, tetracycline-regulated transactivator; HA, hemagglutinin.
sequenced the wild-type CHO β-tubulin gene (GenBank™ accession number AF120325) and then used primers in the intron and 5'- and 3'-untranslated regions to amplify the coding sequences from mutant cell DNA. Sequencing was carried out using 2 distinct approaches. In the first, amplified DNA was directionally cloned into M13mp18 or M13mp19. Multiple individual plaques for each mutation were isolated, and the sequencing reactions for each deoxyoligonucleotide were loaded in adjacent wells of the sequencing gel as described previously (18). Because CHO cells are diploid, mutations were easily identified as changes affecting half of the six to eight plaques that were isolated from each polymerase chain reaction amplification. Polymerase (PiA, Stratagene, La Jolla, CA) errors, on the other hand, were rare and only affected one of the six to eight plaques. A second method involved direct sequencing of the polymerase chain reaction amplified DNA using an ABI model 310 automated sequencer (Perkin-Elmer Corp.). In this case, mutations were detected as coelution of 2 nucleotides from the capillary column when sequenced in both the forward and reverse directions. Mutations were confirmed by repeating the sequencing on freshly amplified DNA and by digesting the polymerase chain reaction-amplified DNA whenever a restriction enzyme site was gained or lost.

Construction of Plasmids—CHO C31 cDNA (GenBank™ accession number U08342) (19, 20), modified to express a 9-amino acid hemagglutinin antigen (HA) tag at the C terminus of β-tubulin (21), was used for all transfections. To obtain regulated expression, pcDNA3 (Invitrogen, Carlsbad, CA) was modified to incorporate the features of a tetracycline regulated vector system (22), and the new plasmid was named pTOPneo. A 1.5-kilobase pair HindIII/NotI fragment from plasmid BlskHAβ (21) containing the entire HAβ1-tubulin coding sequence was cloned into the unique HindIII/NotI sites of pTOPneo to create pTOPneoHAβ. This construct was used for all transfections and for site-directed mutagenesis to create mutant HAβ1-tubulins. A second plasmid carrying the coding sequence of the tetracycline-regulated transactivator (tTA) was made by first replacing the neomycin resistance gene of pTOPneo with the puromycin resistance gene from the vector pPUR (CLONTECH), to create pTOPpuro. The sequence for tTA was then added by cloning a 1-kilobase pair EcoRI/BamHI fragment from the plasmid pHU5-15-1 (22) into pTOPpuro to create pTOPpuro-tTA.

Transfection—Plasmid DNA was isolated using the QIAfilter Plasmid Maxi Kit (Qiagen Inc., Santa Clarita, CA) and transfected into CHO cell line tTApuro 6.6, obtained by transfecting wild-type CHO cells with pTOPpuro-tTA. Transfections were carried out using LipofectAMINE (Life Technologies, Inc.) and 1 µg of plasmid DNA according to the manufacturer’s instructions except that 1 µg/ml tetracycline (Sigma) was included at each step to inhibit expression of the cDNA until the time of analysis. Stable transfectants were isolated and maintained in medium containing 2 mg/ml G418 (Life Technologies, Inc.) plus 1 µg/ml tetracycline or 0.2 µg/ml paclitaxel with no tetracycline.

Electrophoretic Procedures—Preparation of samples for one- and two-dimensional gel analysis has been described previously (23, 24). For Western blot analysis, proteins were electrophoretically transferred onto a nitrocellulose membrane (25) and probed with a mixture of mouse monoclonal antibodies to β-tubulin (Tub 2.1, 1:2,000 dilution, Sigma) and actin (C4, 1:5,000 dilution, ICN). This was followed by incubation in peroxidase-conjugated goat antimouse IgG (1:2,000 dilution, Cappel Laboratories, Cochranville, PA) and detection by chemiluminescence (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) using the manufacturer’s instructions.

Immunofluorescence—Cells were grown on glass coverslips to approximately 70% of confluence and fixed in methanol (−20 °C) for at least 10 min as described previously (21). The primary antibody used for most experiments was mouse monoclonal 12C15 (Roche Molecular Biochemicals), specific for the HA tag. This was followed by fluorescein-conjugated goat antimouse IgG (Cappel). For double-label experiments, mouse monoclonal 6-11B-1 (Sigma), specific for acetylated α-tubulin, was added together with rabbit antibody HA11 (Berkeley Antibody Co., Richmond, CA), specific for the HA tag. This was followed with a mixture of goat affinity purified and cross-absorbed antibodies consisting of Oregon Green-conjugated antirabbit IgG and Rhodamine Red-X-conjugated antimouse IgG (both from Molecular Probes, Inc., Eugene, OR). Photographs were taken on TMX 400 film (Eastman Kodak), using an Optiphot microscope equipped with epifluorescence and a 40X fluor objective (Nikon Inc., Melville, NY).

Results

Paclitaxel-resistant Cell Lines with Altered β-Tubulin—Our laboratory has isolated a large number of paclitaxel-resistant CHO cells with diminished microtubule assembly (14, 16, 27). For the initial sequencing of these mutants, we focused on six cell lines that exhibited alterations in the two-dimensional gel migration of β-tubulin, but we also included seven additional cell lines with no such alterations. Their two-dimensional gel patterns are summarized in Fig. 1. The seven mutants with an altered two-dimensional gel pattern resembled wild-type cells in displaying a single spot for all the expressed forms of β-tubulin (Fig. 1A). The remaining six cell lines with an altered two-dimensional gel pattern fell into two groups. Many displayed an additional β-tubulin spot with a more acidic isoelectric point (arrowhead, Fig. 1B), but strain 11-3 had an additional β-tubulin spot with a more acidic isoelectric point (arrowhead, Fig. 1C). For the mutants with the gel pattern in Fig. 1B, the direction and magnitude of the shift from the wild-type position is consistent with a single charge difference as would be expected for the substitution of a basic for a neutral
Table I

Summary of β-tubulin mutations in paclitaxel-resistant CHO cells

| Cell line | Sequencea | β-Tubulin alteration | Tubulin in polymerb | Resistant or dependentc |
|-----------|-----------|----------------------|---------------------|-------------------------|
| Wild-type | -ACTCTCAAGCTCACCC- | None | 38 |
| Tax-1–4  | -ACTCTCAAGCTCACCC- | L215H | 22 | R |
| Tax-2–4d | -ACTCTCAAGCTCACCC- | L215H | 15 | D |
| Tax-4–9  | -ACTCTCAAGCTCACCC- | L215H | 22 | R |
| Tax-1–19 | -ACTCTCAAGCTCACCC- | L215H | 29 | R |
| Tax-18   | -TCTTTGAAGCTCACCC- | L215F | 15 | D |
| Tax-6–21 | -TCTTTGAAGCTCACCC- | L215F | 15 | D |
| Tax-6–9  | -TCTTTGAAGCTCACCC- | L217R | 31 | R |
| Wild-type | -AACCACCTCCTCTCG- | None | 38 |
| Tax-11–3 | -AACCACCTCCTCTCG- | L228F | 17 | D |
| Tax-2–5  | -AACCACCTCCTCTCG- | L228H | 19 | D |

a For the upper series of mutants, codons 215 and 217 are underlined. For the lower series, codon 228 is underlined. Mutations are indicated in bold letters.
b The fraction of total tubulin assembled into microtubules was determined as described in Ref. 13.
c R, cells are resistant to paclitaxel and grow without the drug. D, cells are resistant to paclitaxel but also require the continuous presence of the drug for cell division.
d Although this cell line has the same mutation as Tax-1–4 and Tax-4–9, its properties, including paclitaxel dependence, differ significantly from the other mutants. We suspect that this cell line may have a second mutation in another gene because of its extremely low reversion frequency (16).

Amino acid or a neutral for an acidic amino acid. The direction of the shift in Tax-11–3, on the other hand, suggests the substitution of an acidic for a neutral amino acid or a neutral for a basic amino acid. Prior work has demonstrated that diploid CHO cell β-tubulin is composed of 70% β1, 25% β4b, and 5% β5 (28, 29). The mutations we have uncovered invariably occur in one of the two allelic β-tubulin gene and therefore affect approximately 1/3 (35%) of the total β-tubulin produced (28). The concentration of mutations in a single gene is probably related to the relative abundance of the β1-tubulin in this cell line. Drug Sensitivity of Paclitaxel-resistant Cells—Resistant cell lines were selected in one step to a single lethal dose of paclitaxel and are approximately 2–3-fold resistant to the drug. Some cell lines are additionally paclitaxel-dependent (14–16). This latter phenotype is easily recognized by a failure of the cells to divide when paclitaxel is omitted from the growth medium and is characterized by a change in morphology to large multinucleated cells (11).4

These and other properties are consistent with a model in which paclitaxel resistance mutations in tubulin destabilize microtubules (12, 30). Direct measurements of the extent of tubulin assembly in mutant cell lines have supported this model (13). Table I summarizes the extent of tubulin assembly in cell lines with β-tubulin mutations. Particularly noteworthy is the observation that paclitaxel-dependent mutants have a lower extent of microtubule assembly than wild-type or resistant cell lines, suggesting that paclitaxel-dependent cells are not fundamentally different from resistant cells. Rather, they simply have mutations in tubulin that are more disruptive to microtubule assembly. Thus, paclitaxel resistance mutations produce a spectrum of alterations in microtubule assembly from minimally disruptive (resistant cells) to highly disruptive (dependent cells).

Leucine Is Frequently Altered in Paclitaxel-resistant Cell Lines—To gain insight into the mutations that destabilize microtubule assembly, β1-tubulin from each of 13 mutant cell lines was sequenced. Four of the seven mutants with a normal two-dimensional gel pattern failed to exhibit an alteration in the β1-tubulin gene. This was an expected result, because we have previously shown that mutations in both α- and β-tubulin confer paclitaxel resistance with equal frequency (16).

Mutations in the β1-tubulin gene from the remaining three mutants, plus the six mutants with an altered two-dimensional gel pattern, are summarized in Table I. We were surprised to find that six of the nine mutations resulted in an amino acid substitution at Leu-215, and in three of these, leucine was replaced by histidine. It is unlikely that the three mutants with a histidine substitution represent sister clones because the cell lines came from independent selections, and the cells have distinctive morphologies. In addition to the His substitution at amino acid 215, only Phe and Arg substitutions were found. The remaining cell lines had L217R, L228F, and L228H substitutions. This clustering of mutations in a small region of β1-tubulin, all affecting leucine residues, is remarkable and suggests a structural motif that may be critical for microtubule assembly. Two of the cell lines exhibited more than a single base substitution in the β1-tubulin gene. Tax-18 has C to T transitions within the same codon (CTC to TTT). Restriction enzyme digestion experiments indicated that both transitions in Tax-18 occurred in the same β1-tubulin allele.5 Tax-11–3 has a G38E substitution (GGA to GAA) in addition to the L228F substitution shown in Table I. The G38E substitution in Tax-11–3 explains the acidic shift observed for the mutant β1-tubulin on two-dimensional gels (Fig. 1C), because the L228F substitution in this mutant is expected to be electrophoretically silent. Transfection experiments (described later) indicate that the G38E mutation does not contribute to paclitaxel resistance, a conclusion that is consistent with the observation that 48 of 48 revertants of this strain retained the G38E mutation as evidenced by retention of the acidic shift in the position of the mutant β1-tubulin on two-dimensional gels (16).

Stable Transfection of Mutant HAβ1-Tubulin cDNA—Although the amino acid substitutions we have uncovered in paclitaxel-resistant mutants predict amino acid changes that are consistent with the mobilities of the altered β1-tubulins on two-dimensional gels (e.g. see Fig. 1), it is possible that other alterations in these cell lines may also contribute to the resistance phenotype. For example, Tax-2–4 (Table I) has a more extreme phenotype than Tax-1–4 or Tax-4–9, even though it has the same L215H mutation. Based on its very low reversion frequency (16), we have long suspected that Tax-2–4 may have a second mutation, but we have not sequenced all of the re-

4 A comparison of the morphologies of paclitaxel-resistant versus -dependent cell lines can be found in supplemental Fig. 2 of the online version of this paper.

5 M. L. Gonzalez-Garay and F. Cabral, unpublished data.
maining tubulin genes to confirm this suspicion. To avoid the ambiguities inherent in trying to assign phenotypes to observed biochemical or genetic changes in the mutant cell lines, we adopted the strategy of recreating the mutations in a cloned cDNA and directly demonstrating that transfection of that cDNA is sufficient to confer paclitaxel resistance. To accomplish this, a chimeric CHO β-tubulin cDNA encoding a 9-aminohexamethaglutinin antigen (HA) epitope tag at the C terminus of the polypeptide (21), was modified by site-directed mutagenesis to introduce the L215H, L217R, and L228F mutations. We circumvented the possibility that overexpression of these mutant genes might be toxic to transfected cells by inserting the altered tubulin cDNAs into a pTOPneo vector that places the gene to be expressed under the control of a minimum cytomegalovirus promoter whose activity requires the binding of a tetracycline regulated transactivator to an upstream bacterial tetO sequence (22). Each of the mutant cDNAs, as well as an unmodified HAβ1-tubulin, was transfected into a CHO strain (tTA/puro 6.6) that was isolated in this laboratory and produces the tetracycline-regulated transactivator in the absence, but not the presence, of tetracycline.

Stable G418-resistant cell lines from each of the transfections were isolated and screened for production of HA-tagged β-tubulin by immunofluorescence. Approximately half of the cell lines for each transfection proved to be positive, and some examples of these are shown in Fig. 2. For each clone, >95% of the cells in the population stained positive for HAβ1-tubulin production. To obtain a more quantitative estimate for the fraction of total β-tubulin represented by the HAβ1-tubulin in each cell line, Western blot analysis with an antibody that recognizes both forms of β-tubulin was carried out (Fig. 3). The HAβ1 transfectant exhibited a very high level of HAβ-tubulin production, resulting in a cell line in which the majority of the endogenous β-tubulin is replaced by the epitope-tagged tubulin at steady state. The HAβ1L217R and HAβ1L228F transfants also had high production of HAβ-tubulin, but the endogenous β-tubulin remained a significant component. The lowest level of HAβ-tubulin was found in the HAβ1L215H transfectant, where it accounted for only a small fraction of the total β-tubulin in the cell. In all four cases, production of HA-tagged tubulin was undetectable by immunofluorescence or Western blot analysis when the cells were grown in tetracycline.

Expression of Mutant HAβ1-Tubulin Destabilizes Cellular Microtubules—Although all transfectants producing wild-type HAβ1-tubulin grew well in the absence of tetracycline, transfectants producing moderate to high levels of mutant HAβ1-tubulin grew poorly. These latter cells frequently exhibited extensive multinucleation during interphase, and there was a clear increase in the number of mitotic cells, indicating a block in mitosis. These observations are consistent with the reduced tubulin assembly measured in the mutants listed in Table I. To further demonstrate that incorporation of mutant HAβ1-tubulin destabilizes cellular microtubules, an HAβ1L215H transfected cell population was selected in G418 and double stained with antibodies to the HA tag and to acetylated α-tubulin. Work in other laboratories has demonstrated that acetylated tubulin is found in the most stable and least dynamic microtubules in the cell (31, 32). We predicted that incorporation of mutant HAβ1-tubulin would cause microtubule destabilization and lead to reduced α-tubulin acetylation. The G418-selected population from cells transfected with HAβ1L215H was approximately 50% positive for expression; a value we have noted in previous transfection experiments (21, 24, 33). Fig. 4A shows two adjacent cells in this population, one of which was positive (small arrow), and the other of which was negative (large arrow), for mutant HAβ1-tubulin production. When the same cells were viewed for acetylated α-tubulin staining (Fig. 4B), a reciprocal relationship was evident. The cell that expressed mutant HAβ1-tubulin had little acetylation of α-tubulin, but the cell that did not express mutant tubulin had abundant α-tubulin acetylation. This result supports the notion that incorporation of mutant HAβ1-tubulin produces less stable microtubules.

Expression of Mutant HAβ1-Tubulin Is Sufficient to Confer Paclitaxel Resistance—Measurement of drug resistance using a standard cloning efficiency assay was complicated by the ob-
Tubulin Mutations and Paclitaxel Resistance

Fig. 4. Cells expressing HAβ1L215H have reduced α-tubulin acetylation. A G418-selected population of cells transfected with HAβ1L215H cDNA was grown 24 h without tetracycline and stained for immunofluorescence with antibodies to the HA tag (A) and to acetylated α-tubulin (B). Small arrows indicate a cell that expressed the mutant HAβ1-tubulin. Large arrows indicate a neighboring cell that failed to express the mutant HAβ1-tubulin. Note that acetylation was greatly reduced in the cell that expressed the mutant HAβ1-tubulin. Bar = 10 μm.

Fig. 5. Mutant HAβ1-tubulins confer paclitaxel resistance. Approximately 100 cells were seeded into replicate wells of 24-well dishes containing the indicated concentrations of paclitaxel (in ng/ml) with (+) or without (−) 1 μg/ml tetracycline. The cells were allowed to grow for 6–7 days and were then stained with methylene blue. The cell lines came from transfections with HAβ1 (A), HAβ1L215H (B), HAβ1L217R (C), or HAβ1L225F (D). Note that all cell lines had a similar sensitivity to the drug when cultured in the presence of tetracycline (no HAβ1-tubulin expression), but only the cells transfected with mutant forms of HAβ1-tubulin exhibited increased resistance to paclitaxel when cultured in the absence of tetracycline.

The drug when mutant tubulin production was induced by growing the cells without tetracycline.

To rule out the possibility that we may have biased the results by examining specific clones of mutant HAβ1-tubulin expressing cells, we also tested the relative abilities of G418 and paclitaxel to select mutant HAβ1-tubulin-positive cells from the total transfected cell populations. We reasoned that paclitaxel should be a powerful agent for selection of mutant HAβ1-tubulin expressing cells if, and only if, the mutant tubulin is capable of conferring resistance to the drug. To test this prediction, aliquots from an HAβ1L215H transfected cell population were grown under six different conditions: normal medium, medium containing 2 mg/ml G418, and medium containing 0.2 μg/ml paclitaxel, each in the presence or absence of 1 μg/ml tetracycline. Using the number of colonies obtained under nonselective conditions (normal medium containing tetracycline) as a control, the relative cloning efficiencies under the various selective conditions are summarized in Table II. The highest cloning efficiency under selective conditions was obtained with G418 in the presence of tetracycline. This was expected because under these conditions, HAβ1L215H cDNA is not expressed, and therefore, transfected cells should be capable of expressing the neomycin resistance gene without suffering negative consequences of HAβ1L215H-tubulin production. The efficiency using G418 under inducing conditions (no tetracycline) was about 40% lower, consistent with the expectation that high expression of HAβ1L215H is deleterious to cell growth. To demonstrate that this is the correct explanation, cells selected under both conditions were compared by immunofluorescence using antibodies to the HA tag. The population selected in G418 under noninducing conditions, but assayed following induction, contained approximately 50% HA-positive cells (Fig. 6A). In stark contrast, cells selected in G418 under inducing conditions were fewer than 10% HA-positive and exhibited weaker fluorescence, indicating that only the cells with lower levels of expression were able to survive (Fig. 6B).
TABLE II
Cloning efficiencies of HAβ1L215H transfected cells under various selective conditions

CHO cell line TApuro 6.6 was transfected with HAβ1L215H cDNA. At 24 h post-transfection, the cells were trypsinized and replated in normal medium (α-MEM), containing 0.2 μg/ml paclitaxel, all either in the presence or absence of 1 μg/ml tetracycline. After 6–10 days (when visible colonies were seen) the cells were stained with methylene blue and the surviving colonies were counted. The cloning efficiency was calculated as the number of colonies obtained under selective conditions (G418 or paclitaxel) divided by the number of colonies obtained under nonselective conditions (α-MEM + tetracycline). Numbers in parentheses are the number of colonies obtained relative to G418 + tetracycline which was arbitrarily set at 100.

| Condition       | +Tetracycline | -Tetracycline |
|-----------------|---------------|---------------|
| α-MEM           | 1             | 1             |
| G418            | 1.5 × 10⁻³ (100) | 7.9 × 10⁻⁴ (60) |
| Paclitaxel      | 1.4 × 10⁻⁴ (1)  | 2.3 × 10⁻⁴ (18) |

FIG. 6. Paclitaxel selects for transfected cells that express mutant HAβ1-tubulin. Cells transfected with HAβ1L215H cDNA were selected for resistance to G418 (A, B) or paclitaxel (C, D) either in the presence (A, C) or absence (B, D) of 1 μg/ml tetracycline. The total resistant cell population was then trypsinized and replated for 24 h in medium without tetracycline or paclitaxel before processing for immunofluorescence with an antibody specific for the HA tag. Arrows in B and C indicate cells that were positive for HAβ1L215H-tubulin expression. Approximately 50% of the cells in A, and 100% of the cells in D were positive for expression. Bar = 10 μm.

Discussion

Selection in paclitaxel under inducing conditions was five times less efficient than in G418 with tetracycline (Table II). This can be explained by the loss of cells that produce too little HAβ1L215H-tubulin to confer resistance or produce too much HAβ1L215H-tubulin to survive. In contrast to the G418 selected cells, virtually all the cells selected in paclitaxel expressed HAβ1L215H-tubulin (Fig. 6D). Thus, paclitaxel is a more stringent agent for selecting mutant HAβ1-tubulin expressing cells than is G418, and this strongly argues that HAβ1L215H-tubulin confers resistance to the drug. Consistent with this interpretation, cells selected with paclitaxel under noninducing conditions formed 18-fold fewer colonies. Cells selected under these conditions grew very poorly and needed to be cultured an additional week in order to obtain enough cells for analysis. The resultant cells (Fig. 6C) were ≈5% positive for HAβ1L215H-tubulin expression and exhibited weaker fluorescence than the cells selected under inducing conditions. Unlike the cells selected for paclitaxel resistance under inducing conditions, which repressed HAβ1L215H-tubulin expression upon tetracycline addition, the HA-positive cells selected under noninducing conditions remained positive regardless of whether tetracycline was present or absent (data not shown). These results indicated that the few cells selected with paclitaxel in the presence of tetracycline consisted of nontransfected cells with borderline paclitaxel resistance (and severe growth problems) and transfected cells with low, unregulated HAβ1L215H-tubulin production.

The preceding data gave us confidence that direct selection of paclitaxel-resistant transfected cells, followed by analysis of transfected gene expression in the selected population, can serve as a rapid and reliable method for testing the ability of various mutations to confer drug resistance. Indeed, when the procedure was repeated three times with HAβ1, or with HAβ1L228F (a random mutation in Tax-11-3) cDNAs, no paclitaxel-resistant cells were obtained despite the selection of thousands of G418-resistant colonies. In contrast, HAβ1L217R and HAβ1L228F cDNAs behaved like the HAβ1L215H cDNA and gave many paclitaxel-resistant colonies, all of which were positive for expression of the mutant gene. We conclude that HAβ1L215H, HAβ1L217R, and HAβ1L228F mutations are sufficient to confer paclitaxel resistance in CHO cells.

The tight distribution of mutations producing paclitaxel resistance was striking and unexpected. All 9 amino acid substitutions changed one of 3 leucine residues that were within 14 amino acids of one another. An alignment of β-tubulin sequences in GenBank™ indicates that the 3 leucine residues are invariant with the exception of an Ile for Leu substitution at amino acid 217 in Schizosaccharomyces pombe. Moreover, the conservation of leucines at 215 and 228 extends to α- and γ-tubulin (34). The results suggest that the 3 residues play an important role in microtubule assembly and in the mechanism of action of paclitaxel.

The high incidence of mutations in one region of the β-tubulin gene in paclitaxel-resistant mutants is not likely to be the result of a mutational "hot spot" for the following reasons: 1) A sequence comparison between β1-tubulin from our laboratory strain of CHO cells and a β1-tubulin cDNA we isolated from a CHO library that was produced in a different laboratory, revealed 77 randomly dispersed nucleotide differences within the coding region (20). None of the nucleotide changes, including a change in codon 228 from CTC to CTG, affected the amino acid sequence. 2) Tax-11-3 has a second mutation (G38D) at a distant location that does not contribute to the paclitaxel resistance phenotype. 3) One mutation that confers Coleclid resistance and five that prevent β-tubulin assembly into microtubules are distant from the 215–228 codons.

Because all 3 mutated leucines in CHO β1-tubulin use a CTC codon, 6 possible amino acid substitutions are permitted by single base mutations. Of these, only 3 (His, Phe, Arg) were actually recovered; furthermore, they were recovered multiple times. Although the number of mutants analyzed is limited, this result could imply that the remaining amino acid substitutions (Pro, Val, Ile) produce mutant tubulin that is 1) assembly-competent but minimally disruptive to microtubule structure and therefore unable to confer paclitaxel resistance, 2) assembly-competent but too disruptive to microtubule structure for the cells to survive selection, or 3) assembly-incompetent and therefore unable to confer paclitaxel resistance. In this regard, it should be noted that substitutions of hydrophobic (Phe) or charged (Arg, His) amino acids can confer resistance. This suggests that it may be the size of the substitution rather than its charge or polarity that destabilizes the microtubules. By this criterion, Val and Ile may not perturb the structure sufficiently to confer resistance. Although Pro should

6 F. Cabral, unpublished data.
cause a significant perturbation, it may cause too large a disruption of microtubule structure, or it may cause the protein to become assembly incompetent; both possibilities would prohibit the mutation from being recovered during selection. Alternatively, it is possible that further analysis will reveal that the need for Leu at positions 215, 217, and 228 is absolute and that virtually all substitutions will perturb microtubule assembly sufficiently to confer paclitaxel resistance. This interpretation is supported by the fact that Leu is almost invariant at these positions among all sequenced α-tubulins. Further site-directed substitutions will be necessary to test these predictions.

The recent publication of the electron crystal structure of tubulin (35) indicates that mutations in codons 215 and 217 are in a loop connecting helices H6 and H7, while the 228 mutation falls within the H7 helix itself. This is the same region (residues 217–231) that was shown to be photocross-linked to 2-(mazidotiazolyl)paclitaxel (36), but is distinct from residue Leu-275 that forms the main interaction with the taxane ring (35) and is distinct from residues F270 and A364 identified as part of the paclitaxel binding site based on genetic studies (10). Although the mutations we identified are close to the region implicated in binding paclitaxel, a number of observations argue that altered paclitaxel binding is not responsible for drug resistance in our mutants (12). For example, β-tubulin is reported to contain the paclitaxel binding site, yet mutations conferring paclitaxel resistance occur with equal frequency in both α- and β-tubulin and both groups of mutants exhibit similar properties (16). Many paclitaxel-resistant mutants require the drug for cell division and therefore must clearly retain the ability to bind the drug. Also, paclitaxel-resistant mutants frequently exhibit increased sensitivity to drugs such as colchicine and vinblastine that bind to distinctly different sites. Instead of altered drug binding, we favor a mechanism in which paclitaxel binding alters the conformation or position of the loop connecting H6 and H7 of β-tubulin in such a way as to facilitate and stabilize subunit-subunit interactions important in the formation of microtubules. Recent fitting of the crystal structure of tubulin to a lower resolution map of the microtubule is consistent with participation of the H6-H7 loop in both longitudinal and lateral contacts (37). The mutations we have identified in leucines 215, 217, and 228 could potentially counteract the effects of paclitaxel by weakening those same interactions directly or by preventing or mitigating the putative conformational change resulting from drug binding. Because the mutations appear to destabilize microtubule assembly in the absence of any drug (Table I and Ref. 13), a direct effect on subunit-subunit interactions appears the more likely possibility.

Because other mechanisms have been proposed to account for paclitaxel resistance in various cell lines, it is worth commenting on possible reasons that assembly mutations occur at such high frequency in CHO cells. Although we have demonstrated previously that CHO cells selected for resistance to colchicine and to vinblastine have a high incidence of the mdr phenotype (38), selections for paclitaxel resistance primarily yield tubulin assembly mutations (16). This difference in frequency could result from different affinities of the drugs for the P-glycoprotein involved in pumping drugs out of mdr cells, but in our view is more likely to result from the fact that mutations in tubulin that destabilize microtubule assembly are relatively common. On the other hand, mutations in tubulin that enhance microtubule assembly, as would be needed for resistance to colchicine or vinblastine, should be relatively rare.

The preceding argument could explain why tubulin mutations are more common in paclitaxel-resistant compared with colchicine- or vinblastine-resistant cells, but does not explain why others have reported mdr as the major mechanism of resistance to paclitaxel (reviewed in Ref. 5). To understand the cause for this difference, it is important to examine the means by which the resistant cells were obtained. In most other studies, multiple step selections were carried out, yielding cells with very high levels of resistance to paclitaxel. Such procedures bias the types of mutations that are ultimately recovered. Mutations in, or amplification of, P-glycoprotein are not detrimental to the growth of cells in culture and thus are retained when selecting for high levels of resistance. In contrast, mutations in tubulin are very likely to affect cell survival if they are too severe and thus would be lost (or at least not predominate) in any selection to high levels of resistance. Since we used single-step selections yielding cells with only 2–3-fold resistance to paclitaxel, there was less bias against the isolation of tubulin mutations compared with multistep procedures. In support of this explanation, selection of human lung carcinoma cells for paclitaxel resistance in a single step also yielded a cell line with altered tubulin rather than mdr (39).

In addition to cells with tubulin assembly mutations or altered P-glycoprotein mediated mdr, human ovarian cell lines with mutations in β-tubulin that may affect paclitaxel binding have been described recently (10). We have not identified similar mutants in our single step selections and have long argued that such mutations should not occur at high frequency in mammalian cells because drug binding mutations are recessive and mammalian cells are diploid for expression of multiple tubulin genes (40). Indeed, the paclitaxel-resistant human ovarian cells were gradually selected to higher levels of resistance than can normally be obtained in a simple one-step procedure and were 24-fold more resistant than the unselected cells. Furthermore, the authors found that both mutants were functionally hemizygous; i.e. only the mutant, but not the wild-type, allele was expressed. Because other β-tubulin isotypes were only expressed at low levels in this cell line, the resistant cells expressed the mutant polypeptide as the predominant β-tubulin species (10). Thus, at least two changes were required to obtain the drug binding phenotype, confirming that such changes should only occur at relatively low frequency.

Finally, a number of laboratories have reported changes in β-tubulin isotype expression that correlate with the acquisition of paclitaxel resistance in various cell lines (6–9). Again, however, these cells were selected in multiple step procedures that may have introduced bias into the kinds of mutants that survived selection. Furthermore, it has not yet been convincingly demonstrated that the altered β-tubulin isotype expression reported in these cell lines is responsible for the drug resistance phenotype. As previously pointed out (10), it is possible that the multiple-step procedures used in those studies enriched for cells that amplified a minor isotype carrying a tubulin mutation that is actually responsible for conferring the resistance. Resolution of these issues awaits manipulation of tubulin isotype ratios by transfection, followed by measurement of paclitaxel resistance in the transfected cells. As a first step in this direction, we have recently found that overexpression of each of three different β-tubulin isotypes (β1, β2, and β4b) is insufficient to confer paclitaxel resistance in CHO cells.8

The mutations we have described appear to be capable, by themselves, of conferring paclitaxel resistance: transfection of

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8 Blade, K., Menick, D. R., and Cabral, F. (1999) J. Cell Sci. 112, 2213–2221.
an HA-tagged β1-tubulin cDNA containing mutations at any of the three leucine residues was sufficient to confer resistance in a wild-type CHO cell line. On the other hand, expression of HA-tagged β1-tubulin cDNA lacking any mutations, or containing an irrelevant mutation (G38D), had no effect on paclitaxel resistance. Alternate explanations for paclitaxel resistance, e.g., altered expression of specific β-tubulin isotypes secondary to diminished microtubule assembly caused by mutant tubulin, are both more complicated and unable to explain all the data, most notably, the existence of paclitaxel-dependent mutants.

In contrast to the original mutants in which altered β-tubulin accounts for approximately 35% of the total, transfected cells are more variable in their production of mutant tubulin and this leads to greater heterogeneity in their response to paclitaxel. Cells in the population that stained less brightly with antibodies to the HA tag grew well in the absence of paclitaxel, but the cells that stained very brightly became multinucleated when paclitaxel was removed from the growth medium. These observations are consistent with a model we proposed earlier suggesting that tubulin mutations causing paclitaxel resistance produce varying destabilization of microtubule assembly, with only the most severe mutations causing paclitaxel dependence (30). We now further demonstrate that the level of expression of mutant β-tubulin can cause varying destabilization of microtubule assembly with higher expression resulting in paclitaxel dependence. This may explain why the L217R mutation, which is not associated with paclitaxel dependence in the original mutant, is able to impart a paclitaxel-dependent phenotype on a subpopulation of the transfected cells. Similar observations have been reported previously for the creation of a Colcemid-dependent cell line by transfection of DNA from Colcemid-resistant cells into wild-type CHO cells (41). This line of reasoning suggests that the severity of a mutation, and its ultimate effects on microtubule stability and paclitaxel resistance, depends not only on the nature of the mutation, but also on the level of expression of the mutant allele in a given cell line.

Our analysis has thus far been limited to mutations in β-tubulin; but we know from two-dimensional gel analysis that mutations in α-tubulin are equally prevalent in paclitaxel-resistant cells (16). It is tempting to speculate that the α-tubulin mutations able to confer paclitaxel resistance will also cluster in a few residues, and this will be a focus of future studies.

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