Paclitaxel-dependent mutants have severely reduced microtubule assembly and reduced tubulin synthesis

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Summary

A subset of mutant cell lines selected for resistance to the antitumor drug paclitaxel are unable to progress normally through mitosis unless the drug is present in the growth medium. Without paclitaxel the cells form defective spindles, undergo aberrant mitoses, fail to complete cell division and eventually die. Analysis of these drug-dependent cells revealed a low amount of microtubule polymer and less tubulin production than wild-type cells. Ribonuclease protection experiments indicated that the decreased tubulin protein was due to decreased tubulin mRNA. Enhancing microtubule assembly by treating the cells with paclitaxel, restored tubulin to levels comparable with those of paclitaxel-treated wild-type cells, which demonstrated that the drug-dependent cells do not have a permanent impairment in their capacity to synthesize tubulin. Paclitaxel-resistant (but not dependent) cells have a smaller reduction in microtubule polymer with little or no decrease in tubulin production, whereas colcemid-resistant cells have increased microtubule assembly but also exhibit little or no change in tubulin production. Finally, a mutant cell line producing an unstable β-tubulin protein has normal growth as well as normal synthesis and polymerization of tubulin, despite an approximately 30% decrease in steady state tubulin content. These studies establish a lower limit of tubulin assembly needed for cell survival and indicate that tubulin assembly must fall below this point to trigger a significant decrease in tubulin synthesis.

Key words: Paclitaxel resistance, Tubulin synthesis, Autoregulation

Introduction

Microtubules are essential cellular structures involved in flagellar and ciliary motility, directed movement of vesicles, organization of the endoplasmic reticulum and Golgi apparatus, and orderly segregation of chromosomes during mitosis. The microtubules exist in a dynamic steady state with tubulin heterodimers from which they assemble (Mitchison and Kirschner, 1984), and maintenance of the dynamic properties appears to be critical for their function (Jordan et al., 1993). The necessity of microtubules for cell survival, coupled with the need to maintain a delicate balance between free and assembled tubulin, makes these structures a good target for many natural toxins including the cancer chemotherapeutic drugs vinblastine, vincristine and paclitaxel.

Heterodimers of α- and β-tubulin are the building blocks for microtubule assembly and, because essential cellular functions are mediated by microtubules, it is anticipated that tubulin should be highly regulated. In fact, production of tubulin appears to be controlled at several levels. The α- and β-subunits are each encoded by a 6-7 member multigene family in vertebrates, and the expression of those genes is regulated in a tissue-specific manner (Sullivan, 1988). For example, βIII-tubulin production appears to be largely restricted to brain and testes; but virtually all tissues contain the more ubiquitous βI-tubulin. Following transcription, tubulin mRNA stability is affected by the state of microtubule assembly. When cells are treated with colchicine, or other drugs that depolymerize microtubules, degradation of tubulin mRNA increases and causes a corresponding decrease in tubulin synthesis. In contrast, when cells are treated with paclitaxel, a drug that enhances microtubule assembly, tubulin mRNA is stabilized and tubulin synthesis increases (Ben-Ze'ev et al., 1979; Cleveland, 1989). It has been proposed that this autoregulatory phenomenon is sensitive to the level of free tubulin in the cell and acts to maintain a steady supply of tubulin for microtubule assembly (Theodorakis and Cleveland, 1992).

Coordinate production of α- and β-tubulin subunits appears to be regulated by a novel mechanism in which α-tubulin translation is inhibited by free α-tubulin but not by α-tubulin that is complexed with the β-subunit (Gonzalez-Garay and Cabral, 1996). In this way, α-tubulin is synthesized only when there are available β-subunits to which it can bind. Finally, tubulin levels can be controlled post-translationally through degradation. Although tubulin heterodimers are very stable (Spiegelman et al., 1977), excess free subunits (Gonzalez-Garay and Cabral, 1995) or defective tubulin proteins have a much shorter half-life (Boggs and Cabral, 1987; Kemphues et al., 1982).

Based on these observations, it is anticipated that tubulin mutations, or treatment of cells with antimitotic drugs, can elicit a complex response that may involve not only changes in microtubule assembly and dynamics, but changes in tubulin production as well. Despite its importance for understanding microtubule regulation, the relationship between tubulin assembly and synthesis is not fully understood. For example, it is not clear whether changes in microtubule assembly elicit

a continuous or discontinuous change in tubulin synthesis; nor is it clear how large a change in microtubule assembly is needed to produce cytotoxicity. Answers to these questions are important not only for understanding the complex regulation of tubulin synthesis and assembly, but also for understanding mechanisms of action for the drugs that affect microtubule assembly.

Prior work from our laboratory demonstrated that a major mechanism by which Chinese hamster ovary (CHO) cells in culture acquire resistance to paclitaxel is through alterations in α- and β-tubulin that diminish the capacity of microtubules to assemble (Cabral, 2000; Cabral and Barlow, 1989). Despite the reduced microtubule assembly observed in paclitaxel-resistant cells, little or no decrease in tubulin synthesis has been found in these mutants, which suggests that the mechanism responsible for autoregulation of tubulin synthesis through modulation of mRNA stability is not activated by small changes in tubulin polymerization. We now report that a subset of mutants that are not only paclitaxel-resistant, but also paclitaxel-dependent for growth, have greatly reduced levels of polymerized tubulin and significantly reduced tubulin synthesis. These studies establish the in vivo limits of microtubule assembly that are consistent with normal microtubule function and cell survival. The data further demonstrate that tubulin synthesis is affected when microtubule assembly falls outside of these normal limits.

Materials and Methods

Growth and labeling of cells

Wild-type CHO cells (Cabral et al., 1980) are a subclone of CHO (Pro-5) (Stanley et al., 1975) and were used to isolate mutant cell lines Tax 18 (Cabral, 1983); Tax 2-4, Tax 5-6, and Tax 9-5 (Schibler and Cabral, 1986); CV 2-8 (Schibler et al., 1989); and 11801 (Whitfield et al., 1986). Cell line 6H2 is a revertant of a colcemid-resistant cell line in which the altered β-tubulin has become assembly defective and unstable (Boggs and Cabral, 1987). All cell lines were maintained in alpha modification of minimum essential medium (MEM) containing 5% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin (all from GIBCO Laboratories, Grand Island, NY) at 37°C in a humid atmosphere containing 5% CO_2. Because cell lines Tax 18, Tax 2-4 and Tax 9-5 are paclitaxel-dependent, the medium for growth of these cells additionally contained 200 ng/ml paclitaxel. Tax 18 R3D is a revertant of Tax 18 that retains paclitaxel resistance but is no longer paclitaxel-dependent and therefore was maintained in medium without drug. Because of a partial dependence on colcemid, strain 11801 was grown in medium supplemented with 50 ng/ml colcemid. Cells were metabolically pulse labeled for 30 minutes in a minimal volume of methionine-free MEM (ICN Pharmaceuticals, Costa Mesa, CA) containing 20 μCi/ml Tran ^{35}S-label (mixture of [^{35}S]methionine and [^{35}S]cysteine, 1000 Ci/mmol; ICN). Steady state labeling was carried out overnight (18-24 hours) in complete medium containing 15 μCi/ml [^{3}H]methionine (80 Ci/mmol, Amersham, Arlington Heights, IL).

Measurement of drug resistance

A plating efficiency assay was used to measure the concentration of drug required to inhibit the growth of each cell line. Approximately 100 cells were seeded into replicate wells of a 24-well tissue culture dish containing increasing drug concentrations. After 6 days, the medium was removed and the cells were stained with 0.05% methylene blue as previously described (Cabral et al., 1980).

Two-dimensional gel electrophoresis

The procedure used is described in detail elsewhere (Cabral and Schatz, 1979). Briefly, cells were metabolically labeled for 30 minutes with [^{35}S]methionine, lysed with SDS gel sample buffer (Laemmli, 1970), and proteins were precipitated with five volumes of cold acetone as previously described (Cabral and Gottesman, 1978). For isoelectric focusing, the protein pellet was resolubilized in urea sample buffer (Cabral and Schatz, 1979) and loaded onto cylindrical 4% polyacrylamide gels cast in 200 μl pipets. The isoelectric focusing gel contained 1% pH 5-7 and 0.2% pH 3-10 ampholytes (Crescent, Islandia, NY). A 7.5% polyacrylamide SDS gel was used for the second dimension.

Tubulin polymerization

A previously described assay was used to measure the extent of tubulin polymerization in various cell lines (Minotti et al., 1991). The procedure involves labeling cells to steady state (16-24 hours) with [^{3}H]methionine, lysing the cells in a microtubule stabilizing buffer (20 mM Tris-HCl, pH 6.8, 0.14 M NaCl, 0.5% Nonidet P-40, 1 mM $MgCl_2$, 2 mM EGTA, 4 μg/ml paclitaxel), separating polymerized from soluble tubulin by centrifugation at 12,000 ***g*** for 5 minutes, adding an [^{35}S]methionine labeled wild-type CHO cell extract to each fraction to act as an internal control, precipitating protein from each fraction with cold acetone, separating proteins by 2D gel electrophoresis, excising β-tubulin from the gels, and calculating the ^{3}H/^{35}S ratio for tubulin from each fraction by liquid scintillation counting. The percentage of total tubulin in the microtubule fraction is then calculated as $\{(^{3}H/^{35}S)p/[(^{3}H/^{35}S)s + (^{3}H/^{35}S)p]\} \times 100\%$, where p is the pellet and s is the supernatant.

Tubulin synthesis and accumulation

Measurements of tubulin synthesis and steady state accumulation have been previously described (Gonzalez-Garay and Cabral, 1995). For synthesis, cells were metabolically labeled with [^{35}S]methionine for 30 minutes, lysed in SDS sample buffer, mixed with a [^{3}H]methionine-labeled wild-type CHO cell extract, acetone precipitated, resolubilized in urea sample buffer and resolved by 2D gel electrophoresis. Relative levels of tubulin synthesis for each strain were compared by measuring the ^{35}S/^{3}H ratio for β-tubulin excised from the gel and normalizing the value by dividing by a similar ratio for actin from the same gel. For accumulation, a similar procedure was followed except that cells were labeled to steady state (16-24 hours) with [^{3}H]methionine, an [^{35}S]methionine-labeled wild-type CHO cell extract was added as an internal control, and ^{3}H/^{35}S ratios were used.

Isolation of RNA

Cells were grown in 100 mm dishes to about 80% confluence, the medium was removed, and 2 ml of Ultraspec-II (Biotecx Laboratories, Houston, TX) was added. The cells were scraped into this solution, transferred to a 17×100 mm polypropylene centrifuge tube, and 1/10 volume of chloroform was added. The two phases were then mixed (Vortex Genie, setting 10), incubated on ice for 10 minutes, and centrifuged at 12,000 ***g*** for 15 minutes. The upper phase was collected, mixed with 0.5 volumes of isopropanol and 0.05 volumes of RNAtrack resin, and centrifuged at 12,000 ***g*** for 2 minutes. The pellet was rinsed with 80% ethanol, air dried and resolubilized in ribonuclease (RNase)-free water. Remaining glass beads were then eliminated by centrifugation.

Ribonuclease protection assay

Labeled antisense riboprobes from CHO *α1-tubulin* and *β1-tubulin* were generated by linearizing the plasmids pRC/HAα1 and

Table 1. Polymerized tubulin in drug-resistant and drug-dependent CHO cell lines

Cell line	Alteration	Phenotype	% Polymerized
11801	βD45Y	$Cmd^{\pm D}$	57±1 (150)*
CV 2-8	αE55K	Cmd^{R}	51±3 (134)
WT		–	38±2 (100)
Tax 5-6	αE22K	Ptx^{R}	28±1 (74)
Tax 9-5	nd	$Ptx^{\pm D}$	21±2 (55)
Tax 2-4	βL215H	Ptx^{D}	12±1 (32)
Tax 18	βL215F	Ptx^{D}	15±2 (39)
Tax 18 +50 ng/ml Ptx			22±2 (58)
Tax 18 R3D	nd	Ptx^{R}	27±1 (71)
6H2	βD45Y/Δ351-362	–	38±2 (100)

Drug-free mutant and wild-type (WT) cells were labeled overnight with [^{3}H]methionine, lysed in microtubule stabilizing buffer and centrifuged to separate soluble from polymerized tubulin. The '% polymerized' represents the fraction of total tubulin found in the pellet. Cmd, colcemid; D, dependent; ±D, partially dependent; nd, not determined; Ptx, paclitaxel; R, resistant. Note that cell line 6H2 contains a β-tubulin gene with two mutations. The first (D45Y) confers colcemid resistance while the second, deletion of amino acids 351-362, occurs in the same allele and makes the tubulin assembly defective and unstable.

*Value in parentheses represents the fraction of total tubulin that is assembled for a given cell line relative to wild-type cells set at 100.

pRC/HAβ1 with the restriction enzymes *Cel*II and *Sap*I respectively, and using them as templates for SP6 RNA polymerase in the presence of α-[^{32}P]UTP. For an internal control, a *Dde*I linearized fragment of the gene encoding mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Ambion, Austin, TX) transcribed with SP6 RNA polymerase was used. The resulting riboprobes were gel purified and used in the RNase protection analyses.

To measure relative levels of mRNA for α1-tubulin, β1-tubulin, and GAPDH, 10 mg of total RNA from wild-type or mutant CHO cell lines were hybridized in solution (40 mM PIPES, pH 6.7, 0.4 M NaCl, 1 mM EDTA, and 80% formamide) to either the α1-tubulin/GAPDH or β1-tubulin/GAPDH ^{32}P-labeled RNA probes described above and the resulting RNA-RNA hybrids were digested in RNase digestion buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA and 300 mM NaCl) with RNase A and T1 for 2 hours at 37°C. The digestion was stopped by the addition of SDS and proteinase K, and the protected fragments were resolved on a 6% denaturing polyacrylamide gel. The dried gel was exposed to X-ray film to obtain an image but was quantified using a Storm phosphoimager (Molecular Dynamics, Sunnyvale, CA), or by cutting out the bands and measuring radioactivity in a model LS2000 liquid scintillation counter (Beckman Instruments, Fullerton, CA).

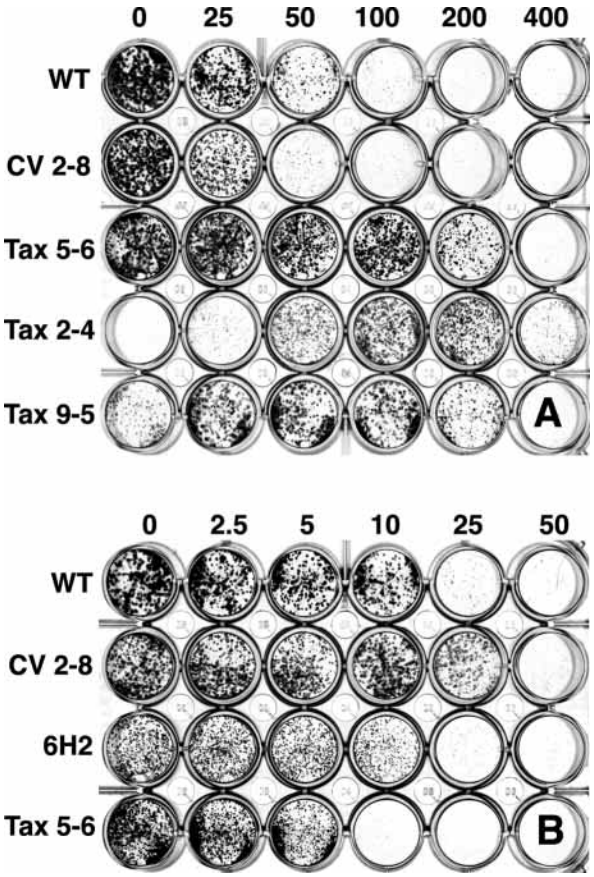

Fig. 1. Sensitivity of wild-type and mutant cell lines to paclitaxel and colcemid. Approximately 200 cells were added to replicate wells of a 24-well dish containing the indicated concentrations of paclitaxel (A) or colcemid (B) in ng/ml. The cells were allowed to grow for 7 days and then stained with methylene blue. Note that compared with wild-type (WT), mutant Tax 5-6 is resistant to paclitaxel but exhibits enhanced sensitivity to colcemid. In contrast, CV 2-8 is resistant to colcemid but more sensitive to paclitaxel. Tax 2-4 is dependent on paclitaxel for growth, while Tax 9-5 is only partially paclitaxel dependent. Strain 6H2 is a revertant of a colcemid-resistant cell line and has regained normal colcemid sensitivity.

Results

Paclitaxel-dependent CHO cells have very low levels of polymerized tubulin

We previously described a sensitive method for quantifying and comparing relative levels of polymerized tubulin among wild-type and drug resistant CHO cells (Minotti et al., 1991). The assay was shown to accurately measure polymerized tubulin at the time of cell lysis, but did not induce in vitro assembly, and could measure subtle changes in microtubule assembly among cells selected for drug resistance or pretreated with agents that perturb microtubules. During the course of those investigations, we found that paclitaxel-resistant mutants have a lower than normal fraction of their tubulin incorporated into microtubules. We now report that paclitaxel-resistant mutants that are also dependent on the drug for their survival have very low levels of polymerized tubulin, which is consistent with a model that we proposed to explain the drug resistance and dependence phenotypes of mutant cells (Cabral, 2000; Cabral and Barlow, 1989). According to this model, microtubules can function sufficiently well to allow cell survival over a range of stabilities or levels of polymerization. However, outside of this range cytotoxicity is observed. Until now, the limits of this normal range of stability were unknown.

Some cell lines selected for resistance to drugs such as colcemid that inhibit microtubule assembly have alterations in tubulin that increase microtubule stability. For example, mutants CV 2-8 (Table 1) and Cmd 4 (Minotti et al., 1991) have an increased fraction (~50%) of polymerized tubulin compared with wild-type cells (~38%). By contrast, cells selected for resistance to paclitaxel, a drug that promotes microtubule assembly (Schiff and Horwitz, 1980), have alterations in tubulin that decrease microtubule stability. An example is Tax 5-6 (Table 1), which has only 28% of its tubulin assembled. All of these cell lines grow equally well in the presence or absence of the selecting drug (Fig. 1). Thus, CHO

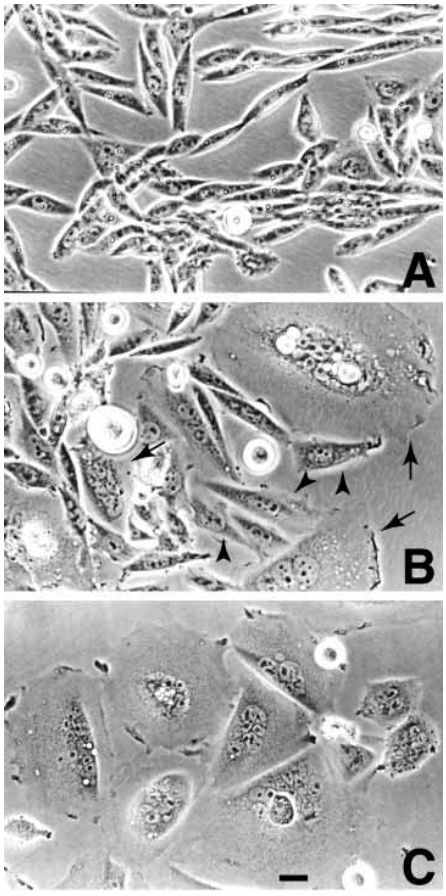

Fig. 2. Morphology of Tax 5-6 (A), Tax 9-5 (B) and Tax 18 (C) in the absence of paclitaxel. Note that Tax 5-6 cells grow normally without drug, whereas Tax 18 cells become large and multinucleated after 2 days without paclitaxel. Tax 9-5 has an intermediate phenotype with both normal (arrowhead) and abnormal (arrow) cells following 2 days of drug deprivation. Bar, 25 μm.

cells can tolerate having as little as 28% and as much as 50% of their tubulin assembled with no obvious effects on growth.

To gain an insight into the limits for how little or how much tubulin assembly is tolerated before cell growth is compromised, we examined mutants that do not proliferate in the absence of the selecting drug. Tax-18 and Tax 2-4 are two previously described paclitaxel-dependent cell lines that are unable to form a functional mitotic spindle, segregrate chromosomes, or complete cell division unless paclitaxel is present in the growth medium, but continue to synthesize protein and DNA at normal rates for at least 60 hours following drug removal (Cabral, 1983; Schibler and Cabral, 1986). As anticipated from the proposed mechanism of resistance, drug-deprived paclitaxel-dependent mutants were found to contain significantly less assembled tubulin (12-15%) than either wild-type cells (38%) or paclitaxel-resistant, but not paclitaxel-dependent, mutants such as Tax 5-6 (28%, Table 1). Thus, 15% assembly is clearly outside the range in which microtubules are able to function, but 28% assembly is within the functional range.

In an attempt to identify the lowest level of polymerization consistent with normal growth, we examined a cell line, Tax 9-5, that has partial dependence on paclitaxel (Fig. 1). These

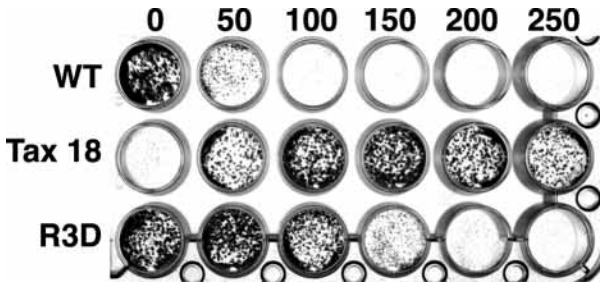

Fig. 3. Paclitaxel sensitivity of Tax 18 and its revertant R3D. Paclitaxel concentrations are in ng/ml. Note that Tax 18 is paclitaxel dependent for growth. R3D was isolated for loss of the paclitaxel-dependent phenotype but retained significant paclitaxel resistance.

cells can grow in the absence of drug, but grow better when drug is present. Morphologically, the culture contains a mixture of normal cells and large multinucleated cells that result from aberrant mitoses when paclitaxel is absent (Fig. 2) (Cabral and Barlow, 1991). This mixed phenotype is seen in this and other similar strains despite repeated subcloning, and suggests that a 21% extent of microtubule assembly is near the transition from functional to non-functional microtubules. Consistent with this interpretation, treatment of Tax 18 with the minimum concentration of paclitaxel (50 ng/ml) that reverses the drug dependence phenotype raised the level of polymerized tubulin to 22%. Moreover, R3D, a revertant of Tax 18 that has lost paclitaxel dependence, but not resistance (Fig. 3), has 27% assembled tubulin.

The upper limit for tubulin assembly consistent with cell survival has been more difficult to obtain because colcemid-dependent cells have not been directly selected. However, colcemid-dependent cells have been previously created by transfecting DNA from colcemid-resistant Cmd 4 into wild-type CHO cells (Whitfield et al., 1986). One of the cell lines from this transfection, strain 11801, was found to express elevated levels of altered β-tubulin and to exhibit poor growth unless colcemid was present in the medium. Analysis of this partially dependent cell line indicated that 57% of the tubulin is assembled (Table 1), suggesting an upper limit to the normal range of tubulin assembly consistent with normal microtubule function.

Together, these results indicate that microtubule function is maintained in CHO cells when the extent of tubulin polymerization falls between approximately 21% and 57% of total tubulin. Outside of these limits, cell growth and survival are compromised. One possible caveat to this conclusion is that the mutations could also be exerting secondary effects on the cells because of changes in microtubule structure, or that mutant cells might activate compensatory mechanisms to counteract the presence of the mutations. To assess the effect of a tubulin reduction independently from the presence of any significant amount of mutant tubulin subunits, we examined strain 6H2. This cell line was derived by selecting revertants of Cmd 4, a colcemid-resistant cell line with a mutation in one copy of its βI-tubulin gene (F.C., unpublished). Revertant 6H2 was found to contain a further 12 amino acid deletion in the mutant protein (F.C., unpublished) that prevented its polymerization into microtubules and dramatically increased its degradation rate, a process we call ‘functional inactivation’

Table 2. Synthesis and steady state accumulation of β-tubulin

Cell line	Normal growth?	Synthesis*	Accumulation*
WT	Yes	100	100
CV 2-8	Yes	112±10	99±3
Tax 5-6	Yes	105±13	94±10
Tax 2-4	No	56±6	53±2
Tax 18	No	60±11	59±3
6H2	Yes	96±6	69±6

To measure tubulin synthesis, cells were labeled for 30 minutes with [^{35}S]methionine and proteins were separated by 2D gel electrophoresis. The radioactivity in tubulin was normalized by dividing by the radioactivity in actin from the same gel. For comparison with wild-type cells, the tubulin/actin ratio for each cell line was divided by the same ratio from wild-type cells and multiplied by 100. Steady state accumulation of tubulin, which reflects both tubulin synthesis and degradation, was measured in a similar manner except that the cells were labeled with [^{3}H]methionine for 16-24 hours. Note that, with the exception of strain 6H2, values for synthesis and accumulation were similar among the various cell lines indicating that there was no enhanced degradation despite the production of altered tubulin. Also note that growth was normal for all cell lines except the paclitaxel-dependent cell lines Tax 2-4 and Tax 18, which fail to divide unless drug is present.

*Relative to wild-type set at 100%.

(Boggs and Cabral, 1987). Because the two copies of the βI-tubulin gene account for 70% of total β-tubulin production in CHO cells (Sawada and Cabral, 1989), rapid degradation of altered tubulin from the mutant allele in 6H2 caused a 30% reduction in tubulin, a value close to the expected 35% decrease if no gene product could accumulate at steady state. However, when analyzed for the percentage of tubulin that is polymerized, 6H2 was found to be normal (38%, Table 1), indicating that this strain has 30% less microtubule polymer and 30% less soluble tubulin than wild-type cells. Because this cell line grows normally, we conclude that a 30% reduction in the level of tubulin in both the polymer and soluble pools is not detrimental to the cells. This conclusion is consistent with the normal growth of mutants such as Tax 5-6, which have also experienced a significant drop in polymerized tubulin. It should be noted that attempts to functionally inactivate a second *β-tubulin* allele by genetic selection failed repeatedly, suggesting that a 60-70% reduction in tubulin is too severe for continued cell survival.

Paclitaxel-dependent CHO cells have reduced synthesis and accumulation of tubulin

Autoregulation of tubulin synthesis has been proposed as a mechanism by which cells sense the level of non-polymerized tubulin and adjust the rate of synthesis to maintain a constant concentration of soluble tubulin for microtubule assembly (Theodorakis and Cleveland, 1992). The existence of mutant cell lines with altered levels of tubulin in the polymer and soluble pools gave us the opportunity to test whether small changes in those levels would affect tubulin synthesis or accumulation. For these experiments, total steady state tubulin was quantified by metabolically labeling cells overnight (1.5-2 generations) with [^{3}H]methionine, resolving tubulin from other cellular proteins on 2D gels, and measuring the radioactivity in β-tubulin, actin and other major proteins by liquid scintillation counting as previously described (Gonzalez-Garay and Cabral, 1995). A summary of the results for some representative cell lines is presented in Table 2. Both

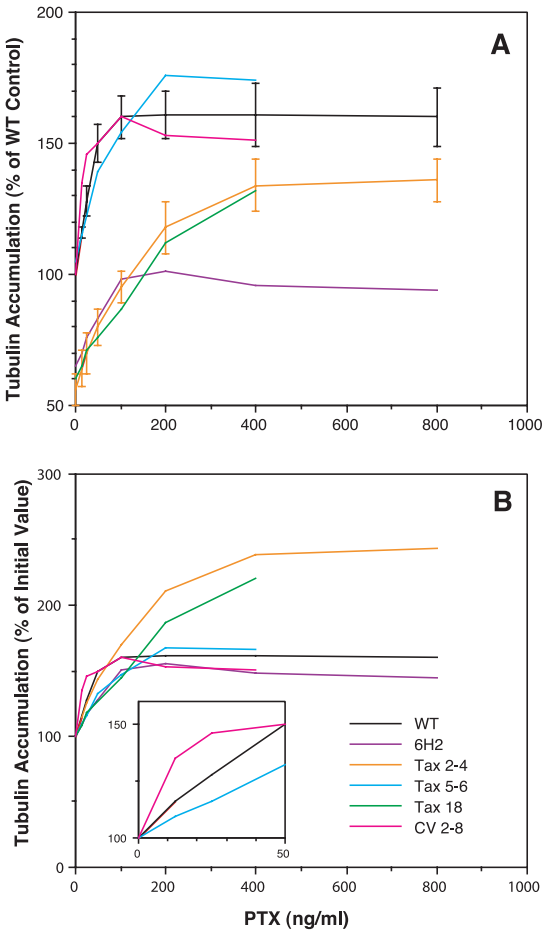

Fig. 4. Effect of paclitaxel on steady state tubulin accumulation. Cells were incubated overnight (16-24 hours) in the presence of [^{3}H]methionine and the indicated concentrations of paclitaxel to measure the amount of protein that accumulated. Following lysis in SDS, the cellular contents were mixed with a constant volume of [^{35}S]methionine-labeled wild-type CHO extract, precipitated with acetone, and resolubilized in urea sample buffer for 2D gel analysis. Spots representing β-tubulin and actin were excised from the gels, solubilized, and analyzed by liquid scintillation counting to determine their 3H/35S ratios. Each isotope ratio for tubulin was normalized by dividing by the isotope ratio for actin in the same sample, and the resulting values were expressed relative to untreated wild-type (WT) cells set at 100% (A) or relative to the zero concentration control for each cell line (B). The values represent the mean from 3-8 independent experiments. Representative standard deviations are shown only for WT and Tax 2-4. The inset in B shows an enlargement of the lower paclitaxel concentrations for WT, CV 2-8 and Tax 5-6.

colcemid-resistant and paclitaxel-resistant mutants were found to have near normal steady state tubulin levels; but cell lines with a paclitaxel-dependence phenotype (Tax 2-4 and Tax 18) exhibited steady state tubulin levels only about 60% as high as wild-type cells. As already discussed, strain 6H2 exhibited a 31% reduction in steady state tubulin consistent with the rapid degradation of an altered β-tubulin subunit that makes up 35% of the total tubulin (Boggs and Cabral, 1987).

The same cell lines were used to measure rates of tubulin synthesis. For these experiments, cells were pulse-labeled with

Table 3. Effect of paclitaxel on tubulin polymerization and accumulation

Concentration (ng/ml)	% Polymerized	Accumulation
0	38±1	100
50	47±3	122±2
100	54±2	133±8
200	59±3	158±12

Wild-type CHO cells were metabolically labeled with [^{3}H]methionine (16-24 hours) in the presence or absence of the indicated concentrations of paclitaxel. The cells were then lysed in microtubule-stabilizing buffer to measure the extent of microtubule assembly (% polymerized) or lysed in SDS to measure total tubulin accumulation, as described in Materials and Methods. Total tubulin in drug-treated cells is expressed relative to untreated cells set at 100.

[^{35}S]methionine for 30 minutes and β-tubulin was quantified as before. The results summarized in Table 2 show that tubulin synthesis is reduced to an extent similar to that of its accumulation, indicating that the changes in steady state tubulin can be fully explained by reduced synthesis rather than enhanced degradation. The sole exception to this was strain 6H2, which had normal tubulin synthesis but reduced steady state accumulation because of enhanced degradation of mutant tubulin subunits.

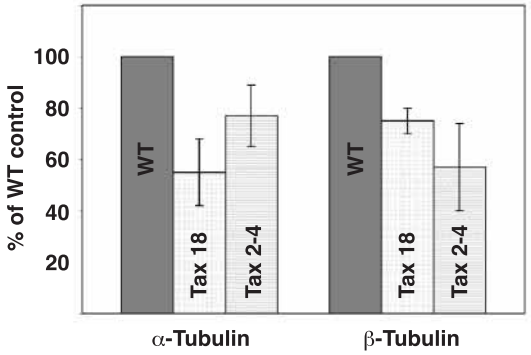

Fig. 5. Quantification of mRNA for α- and β-tubulin. Total RNA was isolated and hybridized to ^{32}P-labeled antisense probes to *α-tubulin* or *β-tubulin*. A second antisense riboprobe to the gene encoding glyceraldehyde-phosphate dehydrogenase (GAPDH) was included in each reaction as an internal control. Following nuclease digestion, the products were separated on a polyacrylamide gel and the protected fragments were identified and quantified on a Storm phosphoimager. The results were also verified by liquid scintillation counting of bands excised from the gel. Relative amounts of tubulin message were calculated by dividing the radioactivity in the tubulin band by the radioactivity in the GAPDH band, and then expressing the results relative to WT cells set at 100%.

Tubulin synthesis is restored by paclitaxel treatment

A number of investigators have shown that drug-induced disruption of microtubule assembly affects tubulin synthesis by an autoregulatory mechanism that alters the degradation kinetics of tubulin mRNA (for a review, see Cleveland, 1989). The decreased tubulin synthesis exhibited by paclitaxel-dependent cells could potentially result from triggering this same mechanism, or it could result from gene inactivation or other mechanisms that permanently reduce transcription. To distinguish between these possibilities, we made use of the well-known observation that paclitaxel enhances the rate of tubulin synthesis by stabilizing (i.e. decreasing the degradation) of tubulin mRNA (Cleveland, 1989). We reasoned that under conditions in which tubulin mRNA is maximally stabilized by paclitaxel, cells that transcribe less tubulin because of inactivation of one of their tubulin genes would continue to synthesize and accumulate less tubulin than wild-type cells under similar conditions. However, cells that have normal transcription but destabilized tubulin message should be capable of a proportionally greater response to paclitaxel stabilization, which should lead to levels of tubulin synthesis and accumulation that approach those of wild-type cells.

To carry out this analysis, wild-type and mutant CHO cells were metabolically labeled overnight with [^{3}H]methionine in the presence of various paclitaxel concentrations and the tubulin accumulated over that period was quantified as already described. The results in Fig. 4 show that wild-type CHO cells exhibited a dose-dependent increase in tubulin accumulation to a maximum of 160% of normal levels. This increase occurred at paclitaxel concentrations that are minimally toxic (e.g. Fig. 1). Tax 5-6, a paclitaxel-resistant mutant with an altered α-tubulin, exhibited a similar increase in tubulin accumulation but the dose response curve was shifted to the right as would be expected for paclitaxel-resistant cells (Fig. 4B, inset). By contrast, Tax 2-4 (Schibler and Cabral, 1986) and Tax 18 (Cabral, 1983), paclitaxel-dependent mutants with altered β-tubulins (Gonzalez-Garay et al., 1999), started with less tubulin in the absence of drug (about 60% of the wild-type level) in agreement with the data in Table 2, but increased to 240% of the initial value, reaching tubulin levels comparable with those of wild-type cells at the higher paclitaxel concentrations (Fig. 4A,B). Similar results have been obtained with other paclitaxel-resistant and paclitaxel-dependent mutants regardless of whether they carry alterations in α- or β-tubulin (data not shown).

The enhanced ability of paclitaxel-dependent cells to respond to the tubulin-synthesis-promoting effects of paclitaxel, strongly argues that their decreased content of tubulin is due to physiological modulation of tubulin synthesis rather than to inactivation of a tubulin gene. Further evidence for this interpretation came from examining the effects of paclitaxel on strain 6H2. As previously mentioned, this cell line has an alteration in one of the β-tubulin subunits that prevents assembly of that subunit into microtubules and causes it to be rapidly degraded. As a result, total tubulin synthesis is normal, but 6H2 has only 70% of wild-type tubulin levels at steady state (Boggs and Cabral, 1987). An increased accumulation of tubulin in response to paclitaxel treatment was also measured in this cell line (Fig. 4); but, in contrast to paclitaxel-dependent cell lines, the maximal increase in tubulin was only 160% of normal and tubulin content never reached wild-type levels even at high paclitaxel concentrations. These results are consistent with the view that paclitaxel-dependent cells express a normal complement of tubulin genes, but that cellular conditions favor more rapid degradation of the tubulin mRNA.

Paclitaxel-dependent cells have reduced levels of tubulin mRNA

To determine whether the decreased tubulin synthesis seen in

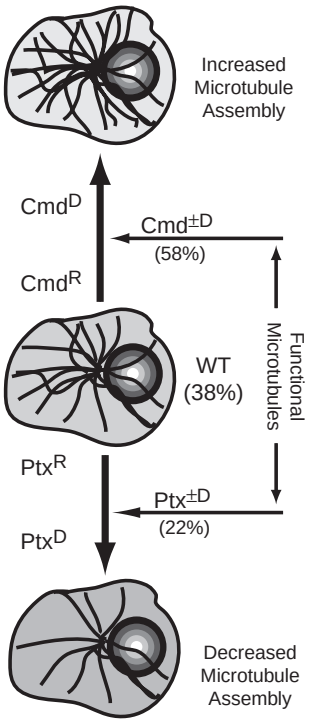

Fig. 6. Mechanism of resistance to antimitotic drugs. Wild-type CHO cells have approximately 38% of their total tubulin in the microtubule fraction (Table 1). Alterations in tubulin that increase microtubule stability lead to increased assembly and resistance to drugs such as colcemid (Cmd) that act to destabilize microtubules. Conversely, alterations that decrease microtubule stability lead to decreased assembly and resistance to drugs such as paclitaxel (Ptx) that act to stabilize microtubules. Alterations that stabilize or destabilize microtubules too much lead to conditional lethal phenotypes such as colcemid-dependence (Cmd^{D}) or paclitaxel-dependence (Ptx^{D}), respectively. Cells with a borderline dependence phenotype (Cmd$^{\pm D}$ and Ptx$^{\pm D}$) define the range of assembly within which microtubules function sufficiently well to allow normal cell growth. The different number of microtubules in each cell is meant to reflect qualitatively the observation that microtubule assembly increases in colcemid-resistant cells but decreases in paclitaxel-resistant cells.

paclitaxel-dependent mutants resulted from lower steady state levels of tubulin mRNA as would be predicted from the autoregulatory mechanism, total RNA from wild-type CHO cells and two paclitaxel-dependent mutants (Tax 18 and Tax 2-4) were analyzed for their ability to protect *α-* and *β-tubulin* probes from RNase digestion. The *α1-tubulin* probe consisted of a 296 nucleotide antisense RNA that produced a 218 bp fragment corresponding to nucleotides 1134-1351 of *α1-tubulin*, and the *β1-tubulin* probe consisted of a 250 nucleotide antisense RNA that produced a 203 bp fragment corresponding to nucleotides 1128-1330 of *β1-tubulin*. For each experiment a 150 nucleotide antisense RNA probe hybridizing to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was included as a control for quantifying the relative amount of α- and β-tubulin mRNA among different cell lines. Following hybridization and RNase digestion, protected probes at the expected sizes were observed and quantified. The results (Fig. 5) indicated that both paclitaxel-dependent cell lines contain lower steady state mRNA levels for α- and β-tubulin compared with wild-type cells, thus accounting for the lower synthesis of α- and β-tubulin in paclitaxel-dependent cell lines (Table 2) and demonstrating that tubulin autoregulation was triggered in these cells.

Paclitaxel increases tubulin production and polymerization in a dose-dependent manner

The ability of tubulin mutations to alter microtubule assembly and affect tubulin synthesis led us to ask whether the effects could be replicated by drug treatment. Wild-type CHO cells were treated overnight with varying doses of paclitaxel and then analyzed for the extent of tubulin polymerization and steady state tubulin levels as already described. The results in Table 3 demonstrate that increases in tubulin accumulation mirror increases in tubulin polymerization induced by drug treatment and that those increases occur at the lowest concentrations of paclitaxel that cause cytotoxicity (Fig. 1A). However, unlike tubulin mutations, paclitaxel treatment produces cytotoxicity and changes in tubulin synthesis within the 'normal range' of tubulin polymerization established by mutant analysis.

Discussion

Resistance to drugs that affect microtubules

Many laboratories have reported the isolation of cultured mammalian cells resistant to paclitaxel and other antimitotic drugs (reviewed by Casazza and Fairchild, 1996). In many cases, the drug-resistant cells were obtained by stepwise selections to very high levels of resistance (100-1000-fold). This procedure resulted in complex phenotypes and a predominance of P-glycoprotein-mediated multidrug resistance as the major mechanism of resistance (reviewed by Cabral, 2000). More recently, selections for cells with moderate levels of resistance (10-20 fold) have been reported and this has led to awareness of tubulin alterations as a frequent mechanism by which cells in culture gain resistance to drugs such as paclitaxel. Although the relevance of tubulin mutations to clinical resistance has recently been called into question (Sale et al., 2002), mutant cell lines have proven useful in making predictions about amino acid residues that may be involved in drug binding (Giannakakou et al., 1997; Giannakakou et al., 2000; Hua et al., 2001) and in mediating potential interactions between tubulin subunits or between microtubules and associated molecules (He et al., 2001; Kavallaris et al., 2001). However, even with cells selected to only moderate levels of resistance, complex phenotypes that include loss of heterozygosity (Giannakakou et al., 1997) and multiple biochemical changes (Kavallaris et al., 2001) have been reported.

Our own studies have used single step selections that give only low resistance (2-4-fold) and have resulted in the isolation of a large series of cell lines with a consistent phenotype (Cabral et al., 1980; Cabral, 1983; Schibler and Cabral, 1986; Schibler et al., 1989). Although we cannot rule out the possibility that multiple changes have occurred in these cells, mutational frequencies, 2D gel analyses, genetic reversion studies and direct transfection of mutant tubulin cDNAs all argue that the indentified alterations in tubulin are sufficient to explain the drug-resistance phenotype (Schibler and Cabral, 1986; Boggs and Cabral, 1987; Gonzalez-Garay et al., 1999; Blade et al., 1999).

We previously proposed a model to explain the drug resistance mechanism in these cells based on the concept that microtubules function normally within a narrow range of microtubule assembly or stability (Cabral et al., 1986). Tubulin mutations that increase microtubule assembly or stability produce resistance to drugs that inhibit assembly; whereas mutations that decrease tubulin assembly or stability produce resistance to drugs that enhance assembly (Fig. 6). Consistent with this model, all paclitaxel-resistant cell lines we have

Table 4. β-Tubulin production in cells with varying levels of tubulin polymer

Cell line	Growth	%P	Synthesis	Accumulation	Polymer*	Soluble†
WT	+	38	100	100	100	100
CV 2-8	+	51	112	99	133	78
Tax 5-6	+	28	105	94	69	109
Tax 2-4	–	12	56	53	17	75
Tax 18	–	15	60	59	23	81
6H2	+	38	96	69	69	69

The table summarizes the ability of each cell line to grow in medium without drug (+, normal growth; –, inability to complete cell division or maintain long-term survival), the percentage of total tubulin assembled into microtubules (%P), the synthesis of β-tubulin relative to wild-type cells set at 100, the steady state level of tubulin relative to wild-type cells (Accumulation), the amount of microtubule polymer relative to wild-type cells, and the amount of nonpolymerized or soluble tubulin relative to wild-type cells.

*Calculated as [%P (mutant) / %P (WT)] × Accumulation (mutant).

†Calculated as [%S (mutant) / %S (WT)] × Accumulation (mutant), where %S=100–%P.

examined have diminished microtubule assembly, while all colcemid-resistant cell lines have increased microtubule assembly (Minotti et al., 1991). We have now further demonstrated that paclitaxel-dependent cells have even lower levels of microtubule assembly compared with cells that are paclitaxel-resistant but not paclitaxel-dependent; and that a colcemid-dependent cell line has higher levels of microtubule assembly than cell lines that are resistant to the drug but not dependent. Thus, drug-dependent cells contain tubulin mutations that exert greater effects on microtubule assembly than the mutations present in resistant cell lines. Indeed, mutations that confer drug-dependence affect microtubule assembly to such an extent, that microtubule function is significantly disrupted and cells are unable to survive unless the selecting drug is present to counteract the effects of the mutation. By measuring relative amounts of microtubule polymer in these mutant cells, we have, for the first time, quantified how severe changes in microtubule assembly must be to produce a drug-dependent phenotype.

The exact mechanism by which mutations in tubulin genes affect microtubule assembly is not known. Many of the alterations in β-tubulin that confer paclitaxel resistance cluster in or near a loop that connects helices 6 and 7 of the protein (Gonzalez-Garay et al., 1999). This loop is situated to potentially play a role in longitudinal or lateral interactions between subunits forming the microtubule lattice (Nogales et al., 1999). Other alterations occur in various regions of α- or β-tubulin subunits, but again could be causing structural changes that perturb subunit interactions to strengthen (colcemid-resistance mutations) or weaken (paclitaxel-resistance mutations) the microtubule lattice. Consistent with this interpretation, immunofluorescence microscopy of paclitaxel-dependent cells reveals a sparse microtubule network and defective mitotic spindles (Cabral et al., 1983). Moreover, a recent study has demonstrated significantly increased dynamic instability in a paclitaxel-dependent cell line (Goncalves et al., 2001).

Microtubule assembly and cell proliferation

The availability of drug-resistant and drug-dependent CHO tubulin mutants has provided an opportunity to determine the tolerance limits for perturbations in microtubule assembly in a mammalian cell line. Drug-resistant cells have small changes in tubulin assembly that produce little or no effect on cell growth and survival, whereas drug-dependent cells have larger changes in assembly that produce clear defects in chromosome segregation and cell division and ultimately cause cell death (Cabral and Barlow, 1991). Poised between drug resistance and drug dependence are cells with intermediate alterations in microtubule assembly that produce partial drug dependence and a significant decrease in cell survival. These latter cells, along with paclitaxel-dependent cells rescued with a minimal concentration of the drug, help to define the limits of how far microtubule assembly can be altered before effects on cell division are encountered. Analysis of these various cell lines suggests that microtubule function is maintained when tubulin assembly is reduced or elevated by up to 45-50%, thereby defining a ‘normal range’ of microtubule assembly that is able to support cell proliferation. Beyond those limits, microtubule function is compromised and cells fail to survive.

Microtubule assembly and tubulin synthesis

These mutants have also allowed us to explore the relationship between microtubule assembly and tubulin synthesis. It has long been known that treating cells with drugs that depolymerize microtubules reduces tubulin synthesis, but that treating with drugs that promote assembly increases tubulin synthesis (Ben-Ze’ev et al., 1979; Cleveland et al., 1981). These changes have been shown to arise from altered tubulin message stability (Caron et al., 1985b; Pittenger and Cleveland, 1985). Although the mechanism by which changes in microtubule assembly are transduced into alterations in tubulin message stability remains unknown, it is believed that cells ‘sense’ the level of nonpolymerized tubulin and adjust message levels to maintain a constant pool of the protein (reviewed by Cleveland, 1989). Thus, it has been proposed that this autoregulatory process acts as a fine-tuning mechanism to ensure appropriate levels of tubulin for assembly (Theodorakis and Cleveland, 1992).

Results from our mutant cell lines demonstrate that cells can tolerate considerable variation in free tubulin levels without evoking changes in tubulin synthesis. Table 4 summarizes the relative levels of polymerized and nonpolymerized (soluble) tubulin in wild-type and mutant CHO cells. In resistant cell populations, soluble tubulin concentrations can vary from 78% to 109% of normal without any accompanying change in tubulin synthesis. Moreover, tubulin synthesis is normal in strain 6H2 despite a 31% decrease in both soluble and polymerized tubulin. The only cell lines that experience a change in tubulin synthesis are Tax 18 and Tax 2-4, paclitaxel-dependent mutants that

assemble too little microtubule polymer to survive when drug is absent. Therefore, mutant analysis argues that, contrary to the expectation for a fine-tuning mechanism designed to maintain a critical concentration of soluble tubulin, changes in tubulin synthesis do not occur despite significant alterations in the concentration of nonpolymerized tubulin. Instead, altered tubulin synthesis is seen only when microtubules become dysfunctional (as assayed by inability of cells to segregate chromosomes and divide).

Most studies using antimitotic drugs to modulate tubulin synthesis have used very high drug concentrations that are clearly cytotoxic, but one study (Caron et al., 1985a) used lower concentrations of colcemid to show a correlation between microtubule disassembly and decreased tubulin synthesis. We have extended these observations to show that low concentrations of paclitaxel increase microtubule assembly and increase tubulin synthesis in a parallel fashion (Table 3). Although the authors of the colcemid study concluded that tubulin autoregulation must be a physiological response because it is triggered by small changes in tubulin assembly, it is now recognized that antimitotic drugs are already cytotoxic at concentrations that have minimal effects on microtubule assembly (Jordan and Wilson, 1998) and we have directly shown that the lowest concentration of paclitaxel that increases microtubule assembly and tubulin synthesis is cytotoxic (compare Table 3 with Fig. 1). Drug treatment differs from mutant analysis in that it is able to elicit changes in tubulin synthesis within the 'normal range' of microtubule assembly (Fig. 6). The reasons for this difference are not yet clear, but we speculate that the binding of a large organic molecule to tubulin may produce pleiotropic effects compared with a more subtle amino acid substitution. Microtubule assembly and function are determined by many factors including tubulin concentration, microtubule dynamics, GTP hydrolysis, and participation of regulatory proteins. It is not unreasonable to expect that drugs and mutations will have differential effects on each of these factors. Drugs produce toxicity at concentrations that alter microtubule assembly to a lesser extent than mutations, suggesting that they may alter additional microtubule properties. In support of this notion, it has been reported that drugs affect microtubule dynamics at very low concentrations (Jordan and Wilson, 1998). While drug treatment and tubulin mutations may differ somewhat in the way they affect microtubule assembly, they both elicit changes in tubulin synthesis only when they become cytotoxic.

The results are consistent with a mechanism in which loss of microtubule function by drug treatment or by mutations produces a stress response that alters the normal turnover of tubulin message. Perturbations that destabilize microtubules also destabilize tubulin message, whereas perturbations that stabilize microtubules stabilize tubulin message. This correlation suggests the existence of a cellular factor that is able to monitor the state of microtubule assembly, but the identity of the factor remains elusive. Early studies suggested that free tubulin itself might be the trigger for changes in mRNA stability (Ben-Ze'ev et al., 1979). This interpretation was directly supported by an experiment in which purified porcine brain tubulin was microinjected into cultured CHO cells and decreased tubulin synthesis was subsequently measured (Cleveland et al., 1983). Although the decreased synthesis was attributed to increased free tubulin, it could also have resulted from altered microtubule assembly or some other non-controlled factor. It is possible, for example, that the microinjected tubulin contained modified subunits that assembled into, and adversely affected the endogenous microtubule network. In support of this interpretation, recent work in our laboratory indicates that expression of brain-specific isoforms of β-tubulin destabilizes the microtubule network in CHO cells (M. Hari and F. Cabral, unpublished). Later studies from Cleveland's laboratory showed no affinity of free tubulin for the cis-regulatory sequence responsible for the autoregulation phenomenon, which demonstrates that free tubulin alone is insufficient to trigger the response (Theodorakis and Cleveland, 1992). In fact, it is difficult to envision how small changes in the pool of a highly abundant protein such as tubulin could trigger changes to maintain the pool at a constant level. Moreover, we have demonstrated by mutational analysis that a 30% change in the pool size of tubulin produces no effect on cell growth or tubulin synthesis.

Although tubulin autoregulation is widespread among vertebrates and even extends to non-vertebrate species, it's physiological function remains unclear. Recently, for example, it was shown that pressure-induced hypertrophy of cardiac tissue resulted in increased microtubule assembly and a persistent upregulation of tubulin by a transcriptional mechanism; there was little or no change in message stability in apparent contradiction to expectations if the autoregulatory mechanism were operating (Narishige et al., 1999). However, the autoregulatory mechanism was shown to be intact and responsive to colchicine treatment indicating that it simply was not activated during the increased tubulin production accompanying hypertrophy. In simpler organisms, Tetrahymena treated with drugs that either promote or destabilize microtubules exhibit increased tubulin synthesis through a transcriptional mechanism (Stargell et al., 1992). However, these cells are capable of modulating tubulin mRNA degradation as demonstrated by the fact that tubulin mRNA is rapidly degraded following the transcriptional upregulation of tubulin message that accompanies deciliation of Tetrahymena (Seyfert et al., 1987). By contrast, changes in tubulin mRNA stability do appear to be triggered during the normal course of embryogenesis in sea urchins (Gong and Brandhorst, 1988). Also, it has been reported that tubulin mRNA is specifically degraded following heat shock in Tetrahymena (Coias et al., 1988). This latter observation combined with the observation that tubulin synthesis is altered in mammalian cells at the lowest cytotoxic drug concentrations, or when the presence of mutant subunits alters microtubule assembly to such an extent that they become non-functional, suggests that autoregulation of tubulin synthesis is triggered by a stress response that may intersect signaling pathways that are used during development. How cells are able to sense microtubule dysfunction and activate signals that affect microtubule message stability will be an interesting area for further investigation.

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