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# Structural and functional alterations in microtubule protein from Chinese hamster ovary cell mutants

**(Colcemid-binding affinity/*in vitro* assembly/tubulin/two-dimensional electrophoresis)**

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**ABSTRACT** We have examined mutant lines of Chinese hamster ovary cells that have increased resistance to the antimicrotubule drug Colcemid. Analysis of the functional properties of purified microtubule protein indicates that increased tolerance to the drug *in vivo* is reflected in altered properties of microtubules and tubulin *in vitro*. In this study, we have examined one series of related mutants and have found different microtubule alterations associated with each selection step. These changes include decreased Colcemid-binding affinity, an altered electrophoretic pattern of tubulin subcomponents, increased resistance to Colcemid inhibition of polymerization *in vitro* and, in one case, a decreased critical concentration for microtubule assembly. Characterized mutants of the class described here will be useful for probing the regulation of microtubule assembly *in vivo*.

Microtubules are a major component of the mitotic spindle and cytoskeleton of eukaryotic cells (1). A characteristic of microtubules is their ability to assemble and disassemble under different conditions. Considerable effort has been expended on elucidating the control mechanisms for microtubule assembly (1–3). Microtubules are formed by polymerization of the globular protein, tubulin, and appear to be regulated by association with a variety of additional proteins, the microtubule-associated proteins (MAPs) (2). Several workers have developed kinetic models to describe the microtubule elongation reaction *in vitro* (4, 5), but *in vivo* regulation of assembly, in particular with respect to initiation, is less well understood (3). We feel that a genetic approach may provide an effective probe of regulatory mechanisms *in vivo*.

To this end, we have isolated mutant clones of Chinese hamster ovary (CHO) cells resistant to Colcemid, with the expectation that these cells would possess altered microtubules (6). We report here the characterization of the properties of microtubules isolated from a related series of Colcemid-resistant mutants. The selection procedures used were designed to avoid the membrane mutants that have reduced drug permeability previously isolated (7, 8). Our results indicate that different alterations in the microtubules of mutant cells contribute to the Colcemid resistance of the series of mutants. We demonstrate here that the purified microtubule protein itself is functionally altered.

## MATERIALS AND METHODS

Cell culture conditions, mutagenesis, and selection procedures have been described (6). The Colcemid-resistant clones are designated by the prefix $CM^R$ and were isolated initially from a clonal line of CHO cells, $E29Pro^+$. The series of clones investigated in this study are related as follows: $E29Pro^+ \rightarrow CM^R7 \rightarrow CM^R79 \rightarrow CM^R795$, where each arrow indicates single-step clonal selection for increased Colcemid resistance (6).

Microtubule protein was isolated from cells grown in monolayer culture, which were washed twice in phosphate-buffered saline and three times in an isotonic buffer of 0.2 M 2-(*N*-morpholino) ethanesulfonic acid (MES), pH 6.6/1 mM $MgCl_2$/1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA)/2 mM 2-mercaptoethanol. To the washed cell pellet was added 0.7 vol of 1 mM $MgCl_2$/1 mM EGTA/2 mM 2-mercaptoethanol/1.7 mM GTP/0.1 mM L-1-tosylamido-2-phenylethyl chloromethyl ketone/0.1 mM *N*-tosyl-L-lysine chloromethyl ketone adjusted to pH 6.6 with NaOH. Cells were broken by ultrasonic cavitation for three 1-min bursts at 0°C (MSE sonicator). The suspension was centrifuged at 81,000 × $g_{max}$ for 40 min at 0°C. Glycerol was added to the supernatant as recommended by Nagle *et al.* (9) to a final concentration of 5 M, and GTP was added to a final concentration of 2.7 mM. The mixture was incubated at 37°C for 25 min and centrifuged at 127,000 × $g_{max}$ for 60 min at 37°C. The pellet was suspended at 0°C in 4 vol of 0.1 M MES, pH 6.6/1 mM $MgCl_2$/1 mM EGTA/2 mM 2-mercaptoethanol/1 mM GTP (suspension buffer). After 40 min at 0°C, the suspension was centrifuged at 81,000 × $g_{max}$ for 30 min at 0°C. The supernatant was adjusted to 2.5 M glycerol, and GTP was added to a total concentration of 2 mM. The mixture was incubated at 37°C for 25 min and layered over 0.5 vol of 4 M glycerol in suspension buffer, also at 37°C. Microtubules were collected by centrifugation through the 4 M glycerol cushion (127,000 × $g_{max}$ for 60 min at 37°C). Three cycles of polymerization and depolymerization were used to complete the purification. Total protein concentration was measured at each stage of the preparation by Lowry's method (10), and active tubulin was measured by the colchicine assay of Borisy (11).

Pure tubulin was prepared from microtubule protein by ion exchange chromatography on DEAE-Sepharose (12). MAPs were eluted with 0.3 M NaCl in suspension buffer, and tubulin was eluted with 0.5 M NaCl in suspension buffer.

Colcemid binding to tubulin was measured by the method of Wilson (13), and binding affinity was calculated from Scatchard plots. Sephadex G-50 columns 1.5 × 30 cm were used to separate bound from unbound [$^3$H]Colcemid. Radiolabeled Colcemid [*ring C, methyoxy*-$^3$H] (9 Ci/mmol; 1 Ci = $3.7 \times 10^{10}$ becquerels) was specially prepared by New England Nuclear.

Microtubule polymerization was monitored by measurement of turbidity at 400 nm. The wavelength was chosen to avoid possible photoisomerization of Colcemid (14). Turbidity mea-

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Abbreviations: CHO, Chinese hamster ovary; MAP, microtubule-associated protein; $CM^R$, Colcemid resistant; MES, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

surements were made in a spectrophotometer equipped with a thermal jacket. Temperature was maintained by water circulators at 0 and 37°C, and rapid temperature changes in the cuvettes (complete in 2 min) were brought about by switching the circulation from one bath to the other. The temperature of the cuvette contents was monitored by a thermistor probe. Microcuvettes used had a volume of 0.15 ml and a path length of 1 cm. Polymerization was induced by changing the temperature from 0 to 37°C. When steady state was reached (30 min), the temperature was returned to 0°C.

The compositions of the microtubule protein mixtures were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (15) and by two-dimensional electrophoresis (16). Gels were stained by a highly sensitive silver staining method (17).

## RESULTS

**Isolation of Microtubule Protein.** We have studied the series of related Colcemid-resistant mutants $CM^R7$, $CM^R79$, and $CM^R795$ derived from the clonal line $E29Pro^+$. The drug-resistant phenotype of these lines has remained stable for more than two years in the absence of selecting agent. This series was chosen because it is amenable to isolation of adequate quantities of highly purified tubulin. The recovery and degree of purification of microtubule protein from the four cell lines is summarized in Table 1. Typically, $4 \times 10^9$ cells gave 1.5 mg of microtubule protein, a recovery of 10–12% of that originally present. Notably, no increase in tubulin content was found in each cell line as resistance to Colcemid was increased. Colchicine-binding activity of the mutant lines is slightly reduced, although purity as determined by electrophoresis was similar to that of $E29Pro^+$. We believe this reduced binding reflects a partial crossresistance to colchicine as well as Colcemid. No further enhancement of specific activity for colchicine binding was observed after an extra purification cycle.

***In Vitro* Polymerization in the Presence of Colcemid.** We have compared $CM^R795$ with $E29Pro^+$ and found that the purified microtubule protein from the mutant polymerizes in the presence of higher concentrations of Colcemid than microtubule protein from the parental line (Fig. 1). This demonstrates that the characteristics of the mutant cellular phenotype are inherent properties of the microtubule protein.

**Colcemid-Binding Affinity.** In previous work, Colcemid resistance had been demonstrated in terms of cell growth in the presence of the drug and by reduced Colcemid-binding affinity of cell homogenates or partly purified tubulin. To determine whether lowered affinity for Colcemid is a property of tubulin itself, or a consequence of other factors present in the extracts, we have prepared tubulin free of MAPs by chromatography on DEAE-Sepharose. Binding of [$^3$H]Colcemid was measured and affinity constants were determined from Scatchard plots (Fig. 2). Free and bound Colcemid were separated on Sephadex columns, which give saturation levels closer to theoretical than the more-rapid filter method. As the Colcemid-binding activity of MAP-free tubulin decays rapidly, the slopes of the Scatchard plots will be biased, depending on the order in which samples are processed. Accordingly, the results shown were determined simultaneously on a battery of matched columns. A progressive decrease in binding affinity was seen for each step of selection (Table 2).

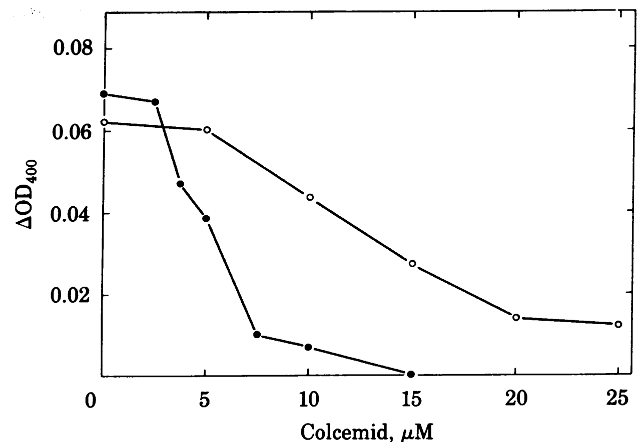


FIG. 1. Polymerization of microtubule protein at 1.0 mg/ml from CHO cells in the presence of Colcemid. •, $E29Pro^+$; ○, $CM^R795$. Polymerization mixtures containing the indicated concentration of Colcemid were kept at ≈18°C for 5 min and then shifted to 37°C to induce polymerization. After 30 min, when polymerization was complete, the temperature was shifted to 0°C, and the turbidity change for depolymerization was measured.

**Polymerization Equilibrium Measurements.** To investigate the functional alteration of microtubule protein, we have measured the critical concentration for tubulin polymerization in the four cell lines (Fig. 3). We were unable to distinguish the microtubule protein of the parent line $E29Pro^+$ from that of $CM^R7$, which was directly derived from the parent. $CM^R79$,

Table 1. Purification of microtubule protein from CHO cell lines

| | Colchicine-binding activity* | | | |
|---|---|---|---|---|
| | $E29Pro^+$ ($4 \times 10^9$) | $CM^R7$ ($6 \times 10^9$) | $CM^R79$ ($5 \times 10^9$) | $CM^R795$ ($4 \times 10^9$) |
| First supernatant | | | | |
| Total activity, cpm | $2.0 \times 10^6$ | $2.1 \times 10^6$ | $1.78 \times 10^6$ | $1.44 \times 10^6$ |
| Specific activity, cpm/mg of protein | 5,565 | 2,060 | 2,926 | 2,983 |
| $C_1S$ | | | | |
| Total activity, cpm | 844,000 | 1,099,677 | 986,615 | 855,163 |
| Specific activity, cpm/mg of protein | 76,730 | 61,918 | 40,501 | 53,784 |
| $C_3S$ | | | | |
| Total activity, cpm | 244,557 | 202,230 | 170,817 | 213,060 |
| Specific activity, cpm/mg of protein | 165,241 | 134,820 | 137,592 | 139,350 |

[$^3$H]Colchicine ($3.8 \times 10^7$ cpm/μmol) was incubated at 5 μM with protein samples for 75 min at 37°C. Under similar conditions, highly purified bovine brain microtubule protein has a specific activity of 167,000 cpm/mg of protein. $C_1S$ and $C_3S$, Supernatants after, respectively, one and three cycles of assembly and disassembly.

* Values in parentheses represent number of cells.

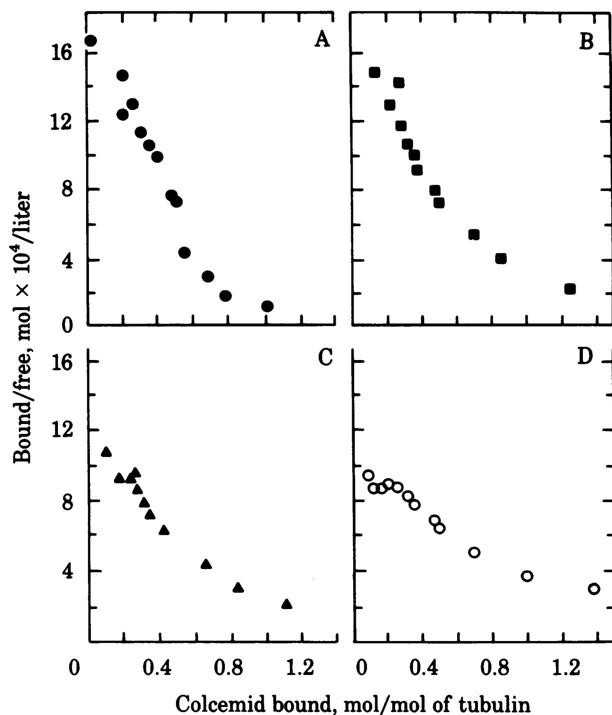


FIG. 2. Scatchard plots of binding of [$^3$H]Colcemid to CHO cell tubulin purified by DEAE-Sepharose chromatography. Protein samples (0.1 mg/ml each) were incubated at 37°C for 40 min. (*A*) E29Pro$^+$; (*B*) CM$^R$7; (*C*) CM$^R$79; (*D*) CM$^R$795. Protein-bound radioactivity was separated from unbound on Sephadex G-50 columns. Binding affinity was calculated (see Table 2) from the slope, taking the first eight points representing the high-affinity binding kinetics. $r^2 \geq 0.99$ for all lines except CM$^R$795 (0.92). A molecular weight of 110,000 for tubulin was assumed.

derived from CM$^R$7, had the same apparent critical concentration as its parents, but the slope of the critical concentration plot was lower. CM$^R$795 had a considerably lower critical concentration than the other cell lines, and the slope was similar to that of CM$^R$79, from which CM$^R$795 was derived. These results were reproducible in replicate preparations.

**Gel Electrophoresis.** Single dimension NaDodSO$_4$/polyacrylamide gel electrophoresis showed similar patterns for all four cell lines. A band corresponding to MAP-2 of bovine brain tubulin (18) was also found consistently but at low intensity (data not shown).

We saw differences between parental and resistant cell lines by two-dimensional electrophoresis with isoelectric focusing as

Table 2. Colcemid resistance and decreased binding affinity

| | E29Pro$^+$ | CM$^R$7 | CM$^R$79 | CM$^R$795 |
|---|---|---|---|---|
| Relative resistance | 1 | 2 | 4 | 9 |
| Colcemid-binding affinity of cytoplasm extract | $1.6 \times 10^5$ | $1.4 \times 10^5$ | $9.7 \times 10^4$ | $4.3 \times 10^4$ |
| Colcemid-binding affinity of DEAE-Sepharose-purified tubulin | $2.5 \times 10^5$ | $2.0 \times 10^5$ | $1.3 \times 10^5$ | $7.1 \times 10^4$ |

Relative resistance was calculated by dividing the concentration of drug required to reduce the colony-forming ability of a cell line to 10% of control (the D10 value) by the D10 value of the parental line E29Pro$^+$. Colcemid-binding affinity ($K_a$) was calculated from Scatchard plots (see Fig. 2).

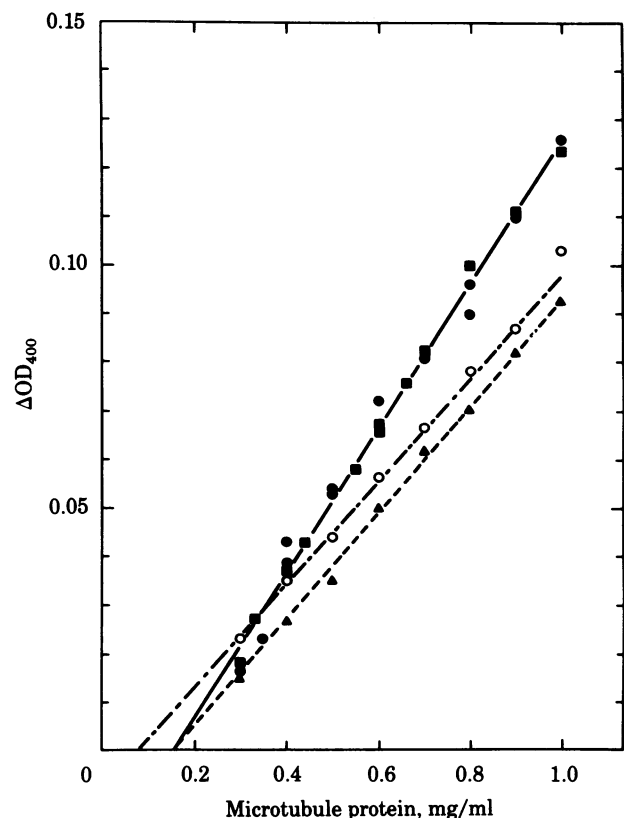


FIG. 3. Critical concentration plots for microtubule polymerization. Polymerization was measured as described in the legend to Fig. 1, except that protein concentration was varied. Colcemid was omitted, and the preincubation step (5 min at 18°C) was omitted. ●, E29Pro$^+$; ■, CM$^R$7; ▲, CM$^R$79; ○, Cm$^R$795. Critical concentration is the intercept on the concentration axis. For each set of points, $r^2 \geq 0.99$. Data for CM$^R$795 were confirmed with another independent preparation. Intercepts are 0.158, 0.158, 0.162, and 0.081 mg/ml for E29Pro$^+$, CM$^R$7, CM$^R$79, and CM$^R$795, respectively.

the first dimension (Fig. 4). Tubulin was resolved into $\alpha_1$, $\alpha_2$, and $\beta$ components, and this was seen for E29Pro$^+$. CM$^R$ lines 7, 79 and 795 showed, in addition, a more basic pair of components that corresponded in apparent molecular weight to $\alpha_1$ and $\alpha_2$. The simultaneous appearance of modified $\alpha_1$ and $\alpha_2$ in the single-step mutant CM$^R$7 suggests that these polypeptides, which differ from each other in apparent molecular weight but not in charge, are not coded on separate genes. Only a small proportion of tubulin was found modified, however, and no increase in the proportion of modified component was apparent in CM$^R$79 and CM$^R$795, which have greater resistance to Colcemid. The new polypeptides have been demonstrated to be tubulin by crossreactivity to monospecific antitubulin antibody (Fig. 5).

## DISCUSSION

Our approach in selecting mutants resistant to antimicrotubule drugs is based on the rationale that cells may have several ways to circumvent the effects of a drug. By this means, we may obtain mutants that have alterations affecting many aspects of microtubule function. Permeability mutants occur with greatest frequency (7, 8); these mutants owe their resistance to the exclusion of drug from the cytoplasm rather than to functional changes in the microtubules themselves. In this study, specific steps were taken to exclude such mutants (6). Permeability

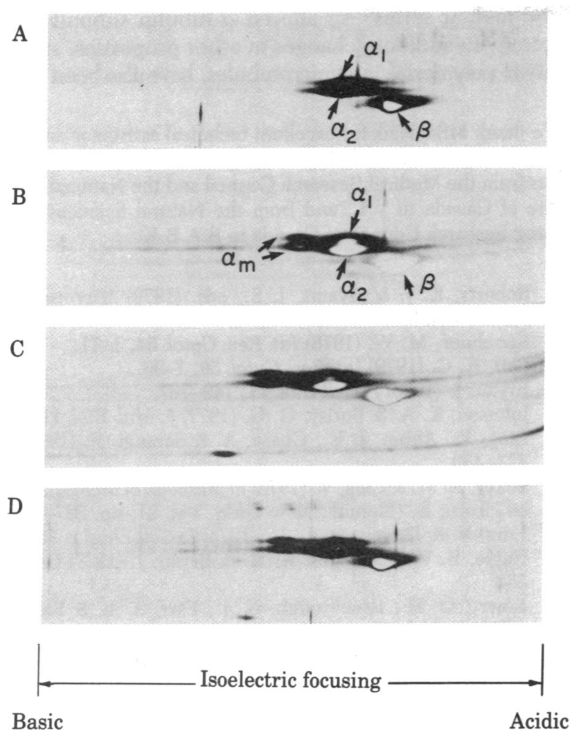


FIG. 4. Two-dimensional electrophoresis of tubulin from CHO cells. Samples (2 $\mu$g) were separated by isoelectric focusing as the first dimension in gels containing Ampholines pH 5–7 and pH 3.5–10 (4:1) and $NaDodSO_4$/electrophoresis as the second dimension. Separated components were revealed by a sensitive silver staining procedure (17). Tubulin present in high concentrations stains as golden spots. Only the portion of the $NaDodSO_4$ dimension containing $\alpha$- and $\beta$-tubulin is included in this figure. (*A*) E29Pro$^+$. (*B*) $CM^R7$. (*C*) $CM^R79$. (*D*) $CM^R795$. The various components of tubulin are indicated; $\alpha_m$ is the altered component.

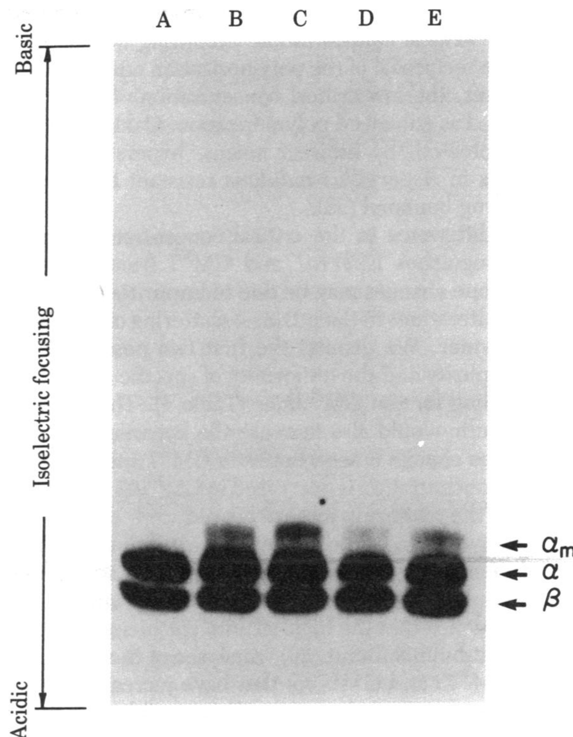


FIG. 5. Radioautogram of CHO cell tubulin separated by isoelectric focusing on a 5% polyacrylamide slab gel. First-dimension conditions were as described in the legend to Fig. 4. Electrophoretic transfer onto nitrocellulose and staining with antibody and $^{125}$I-labeled protein A were as described (19). Affinity-purified monospecific rabbit anti-tubulin antibody was obtained from J. E. Aubin (University of Toronto). Samples were 40 $\mu$g each. (*A*) E29Pro$^+$. (*B*) $CM^R7$. (*C*) $CM^R79$. (*D*) $CM^R795$. (*E*) A second preparation of $CM^R795$.

mutants are also characterized by a broad spectrum of resistance to nonmicrotubule drugs such as daunomycin and puromycin. The mutant clones described here showed their greatest resistance to the selecting drug Colcemid, partial resistance to other antitubulin drugs such as colchicine, and no resistance to daunomycin or puromycin (6). Drug resistance may also be a consequence of gene amplification causing an increase in the quantity of the drug target protein (20), tubulin, in the case of Colcemid resistance. We find no evidence, based on colchicine-binding assays and recovery calculations (Table 1), to suggest that any Colcemid resistance observed may be simply due to increased levels of tubulin.

In this paper, we demonstrate significant alterations in the functional properties of purified tubulin from the mutant cell lines. This is shown most directly by the ability of purified microtubule protein from $CM^R795$ to polymerize in the presence of a 3-fold higher concentration of Colcemid, compared with microtubule protein from E29Pro$^+$. Clearly the *in vivo* phenotype of drug resistance is a property of the microtubules themselves. We consider the altered $CM^R$ lines to be mutants according to commonly accepted criteria for somatic cells (21). The altered phenotype was selected from a clonal line after exposure to a mutagen, it is stably transmitted through succeeding generations (6), and we now show reproducible alterations in a purified gene product. Somatic cell hybrids have also been constructed between $CM^R$ cell lines and Colcemid-sensitive auxotrophs of CHO. Hybrid clones retain the drug resistante but at a reduced level, showing incomplete dominance (6). In related work, it has been shown by immunofluorescence microscopy that microtubules of these mutants are more resistant to breakdown by Colcemid *in vivo* and that vinblastine crystals formed in these cells differ from those in parental cells (22).

The mechanism of action of colchicine, Colcemid, and podophyllotoxin has been described in detail (23–26). It is known that the drug molecules bind to free tubulin dimer first and do not directly inhibit microtubule polymerization (23). The tubulin dimer–colchicine complex that forms then interferes with microtubule assembly (24, 26). This mechanism suggests two ways in which an altered tubulin might escape the effects of Colcemid: lower binding affinity for the drug could reduce formation of the assembly-inhibiting complex, and hyperstabilization of polymerization could counteract the inhibition resulting from the formation of the tubulin–Colcemid complex.

Alkaloid binding to tubulin at the colchicine site is inhibited by MAPs (27). To distinguish possible MAP mutants from alterations to tubulin itself, we have shown that the reduced affinity of tubulin for Colcemid was maintained after the MAPs were removed by DEAE-Sepharose chromatography. The binding affinity for Colcemid decreased progressivley for each selection step in the construction of the mutants, and the degree of reduction in affinity at each step was similar when Colcemid binding to microtubule protein was compared with Colcemid binding to tubulin. These data indicate that a property of tubulin itself has been altered. The affinity constants for Colcemid binding to DEAE-Sepharose-purified tubulin were uniformly higher than those for binding to the tubulin component of cytoplasmic extract, and this observation is consistent with the removal of MAPs from tubulin during purification (27).

Enhanced polymerization has been demonstrated in $CM^R795$

only. This is seen in the critical concentration determination (Fig. 3). The critical concentration, according to Johnson and Borisy, is the reciprocal of the polymerization equilibrium constant (5). Thus, the low critical concentration of CM$^R$795 can be interpreted as enhanced polymerization. Oakley and Morris have also detected, by indirect means, hyperstabilization of microtubules in *Aspergillus nidulans* resistant to the antimicrotubule drug benomyl (28).

Another difference in the critical concentration plots, the slopes, distinguishes E29Pro$^+$ and CM$^R$7 from CM$^R$79 and CM$^R$795. Slope changes may be due to impurities, inactive tubulin, or to alterations in the intrinsic scattering of light per unit mass of polymer. We dismiss the first two possibilities from evidence of purity and the uniformity of specific activity of colchicine binding for the CM$^R$ lines (Table 1). The presence of inactive tubulin would also increase the apparent critical concentration: no change is seen between CM$^R$7 and CM$^R$79, and the critical concentration is decreased in CM$^R$795. We therefore deduce that the scattering intensity is reduced, possibly due to changes in hydration or ion association with tubulin (29).

Two dimensional gel electrophoresis showed structurally altered $\alpha$-tubulin components in the first-step mutant CM$^R$7. There is evidence that the high-affinity colchicine-binding site is on the $\alpha$-tubulin subunit (30). Analyses of the other related mutants (CM$^R$79 and CM$^R$795) that have increased resistance did not show additional alterations. It is possible that structural changes in the tubulins of these lines could be neutral in charge and would not be detected by this system. It could be argued that alterations involving charge differences are more likely to produce functional changes in a protein. However, many so-called "conservative" amino acid substitutions result in substantial changes in secondary structure potential (31), which could affect configurations of binding sites for drugs or for other protein subunits. It is significant that new tubulin spots have appeared without the disappearance of the original spots, and alterations in posttranslational processing of the peptide may be possible. However, sequences of cDNA for chicken brain tubulin do not suggest any major posttranslational processing of the peptide chain other than the COOH-terminal tyrosine of $\alpha$-tubulin (32), which is not yet known to alter any functional property of tubulin. We feel that we can explain our results more readily by proposing the existence of multiple tubulin genes, for which there is evidence in other systems (33–35). Results such as that for CM$^R$7 may then arise because of an actual structural mutation in one of many tubulin genes or by a regulatory mutation that allows expression of an intrinsically Colcemid-resistant tubulin gene that is normally inactive. Experience with many Colcemid-resistant mutants suggests that a single-step mutation rarely results in development of Colcemid resistance by a factor >2 (6). Such small changes in drug resistance are to be expected if a mutation can only alter one of many tubulin genes.

Recently, Cabral *et al.* have reported the isolation of a Colcemid-resistant CHO cell line (36). They observed in the mutant line an additional less acidic $\beta$-tubulin component; however, they were not able to detect an altered drug-binding affinity.

In summary, we have shown that our Colcemid-resistant CHO cell lines contain functionally altered tubulin. A concomitant decrease in Colcemid-binding affinity of the purified tubulin is observed with each step of increased drug resistance in the mutant series. An altered $\alpha$-tubulin subunit has been detected. In addition, changes in other properties, such as the ability to polymerize into microtubules, have also been observed.

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