Kinetic Stabilization of Microtubule Dynamics at Steady State *in Vitro* by Substoichiometric Concentrations of Tubulin−Colchicine Complex[†]

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ABSTRACT: We have analyzed the effects of tubulin−colchicine (TC)-complex on the dynamic instability behavior of bovine brain microtubules at steady state *in vitro* using video microscopy. Incorporation of low numbers of TC-complexes per microtubule strongly suppressed dynamics at the plus ends by reducing the rate and extent of growing and shortening and by increasing the time microtubules spent in an attenuated state, neither growing nor shortening detectably. In addition, TC-complex strongly suppressed the catastrophe frequency and increased the rescue frequency. At low concentrations (0.02−0.05 μM), TC-complex suppressed dynamics without reducing the polymer mass or the mean microtubule length. Such strong suppression of microtubule dynamics by low TC-complex concentrations in the absence of polymer mass changes demonstrates that microtubule dynamics are more sensitive to the actions of TC-complex than the polymer mass. Significant reduction of polymer mass occurred at relatively high TC-complex concentration (>0.05 μM). However, the surviving microtubules were extremely stable. Thus, TC-complex stabilizes microtubules even though the microtubules can transiently depolymerize when TC-complex is added. The data also directly establish that kinetic suppression of dynamics by colchicine at low concentrations is effected by a low number of TC-complexes at the microtubule ends.

Microtubules, major components of the eukaryotic cytoskeleton, are tube-shaped polymers composed of αβ tubulin heterodimers arranged parallel to a cylindrical axis. Microtubules are intrinsically dynamic polymers that display a number of nonequilibrium behaviors both *in vitro* and in cells [reviewed by Wordeman and Mitchison (1994) and Wilson and Jordan (1994)]. One such nonequilibrium behavior that is important in cells is called dynamic instability; a behavior in which microtubule ends stochastically switch between growing and shortening states (Mitchison & Kirschner, 1984; Horio & Hotani, 1986; Walker et al., 1988). At any given time, most microtubules are growing while relatively few are shortening rapidly. Also *in vitro* and in cells at polymer mass steady state, net growing of microtubules in a population can occur at one microtubule end and net shortening can occur at the opposite end, a behavior termed treadmilling or flux (Margolis & Wilson, 1978; Farrell et al., 1987; Hotani & Horio, 1988; Mitchison, 1989). The mechanism responsible for the nonequilibrium dynamics involves the gain and loss of a stabilizing cap at microtubule ends, hypothesized to be composed of tubulin−GTP or tubulin−GDP·P_i.

There is strong evidence that the dynamic properties of microtubules are critically involved in their functions [reviewed by McIntosh & Herring (1991), Wordeman and Mitchison (1994), and Wilson & Jordan (1994)]. For example, rapid microtubule dynamics are involved in chromosome movement during mitosis, which involves dynamic interactions between the kinetochores of the chromosomes and the spindle microtubules. Recent evidence indicates that low concentrations of the antimitotic drug vinblastine inhibit mitosis in HeLa cells at the metaphase−anaphase transition by suppressing the dynamics of spindle microtubules rather than by depolymerizing the microtubules (Jordan et al., 1991, 1992; Wilson & Jordan, 1994). These results have indicated that antimitotic drugs that act by modulating microtuble dynamics may serve as useful probes to investigate the role of microtubule dynamics in cells.

Colchicine is a potent antimitotic drug that has played a fundamental role in elucidation of the properties and functions of tubulin and microtubules since it was first discovered to bind to the subunits of microtubules in cell extracts more than 25 years ago (Borisy & Taylor, 1967a,b; Wilson & Friedkin, 1967). The binding reaction between colchicine and tubulin is unusual, and considerable evidence indicates that the unusual features of the binding reaction are important in determining the potent actions of colchicine on microtubule polymerization. Microtubules have very low or no affinity for free colchicine (Wilson & Meza, 1973; Lee et al., 1974; Skoufias & Wilson, 1992), and in order to produce its potent actions on microtubule polymerization, colchicine must first form a complex with soluble tubulin (Margolis & Wilson, 1977; Skoufias & Wilson, 1992).

The initial step in the reaction between colchicine and tubulin is formation of a reversible preequilibrium complex that is followed by one or more slow steps in which conformational changes in tubulin lead to formation of a poorly reversible final-state tubulin−colchicine complex (TC-complex[1] [reviewed by Hastie (1991)]. Considerable data indicate that tubulin undergoes a conformational change upon binding of colchicine. For example, the binding of colchicine to tubulin quenches the intrinsic tryptophan fluorescence of tubulin (Garland, 1978; Lambeir & Engelborghs, 1981) and perturbs its far-ultraviolet circular dichroism spectrum (An-

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dreu & Timasheff, 1982). In addition, colchicine binding to tubulin strongly increases the intrinsic GTPase activity of tubulin (David-Pfeuty et al., 1979; Andreu & Timasheff, 1982; Perez-Ramirez & Timasheff, 1994), it increases the affinity of $\alpha-\beta$ dimer association (Detrich et al., 1982; Panda et al., 1992), it changes the exposure of sulfhydryl groups in tubulin (Luduena & Roach, 1991), and it induces tubulin to self-assemble into nonmicrotubule polymeric structures (Andreu et al., 1983). Tubulin-bound colchicine is the active form of the drug. Thus, while the nature of the slow conformational changes that occur during formation of the final-state TC-complex is unknown, it seems clear that the conformational changes are involved in the powerful effects of colchicine on polymerization.

Colchicine can inhibit microtubule polymerization substoichiometrically at concentrations that are far below the concentration of total tubulin (Olmsted & Borisy, 1973; Margolis & Wilson, 1977; Margolis et al., 1980; Skoufias & Wilson, 1992). These data have indicated that low concentrations of colchicine inhibit microtubule polymerization by acting at the microtubule ends. It has been known for some time that substoichiometric concentrations of TC-complex only partially depolymerize microtubules (Margolis et al., 1980; Wilson & Farrell, 1986; Skoufias & Wilson, 1992; Vandecandelaere et al., 1994) and that low concentrations of TC-complex can stabilize microtubules against dilution-induced disassembly (Margolis et al., 1980; Farrell & Wilson, 1984; Wilson & Farrell, 1986). These studies have lead to the hypothesis that binding or incorporation of TC-complex at microtubule ends kinetically "caps" or stabilizes the ends. An important feature of the mode of action of colchicine is that TC-complex can form copolymers with free tubulin when microtubules are assembled in the presence of the complex (Sternlicht and Ringel, 1979; Sternlicht et al., 1983; Farrell & Wilson, 1984). Microtubule copolymers can contain relatively large numbers of TC-complexes throughout the polymers. Sternlicht & Ringel (1979) hypothesized that the extensive incorporation of TC-complex throughout the microtubule was responsible for the ability of colchicine to inhibit microtubule polymerization. However, the quantity of TC-complex incorporated into the microtubules depends upon the experimental conditions, and the two hypotheses for how TC-complex inhibits polymerization are not mutually exclusive (Sternlicht & Ringel, 1979; Farrell & Wilson, 1984).

Most previous work on the effects of TC-complex on microtubule polymerization has been performed in bulk solution, and such studies cannot provide insight into how the drug modulates the growing and shortening dynamics at the ends of individual microtubules. Data on the action of TC-complex on the dynamics of individual microtubules were recently reported by Vandecandelaere et al. (1994), who studied the effects of high TC-complex concentrations on microtubule dynamics in a buffer system containing 1 M glycerol. They found that TC-complex inhibited microtubule growing rates and strongly suppressed tubulin dimer exchange, and they suggested a mechanism for the action of TC complex at microtubule ends based upon a lateral cap model. However, some of the actions of TC-complex may have been masked by the glycerol which stabilizes microtubules (Kristofferson et al., 1986; O'Brien & Erickson, 1989), and no experimental data were provided on the shortening rates or on the transition frequencies, parameters that are important in regulation of microtubule dynamics in cells (*e.g.*, Belmont et al., 1990; Gliksman et al., 1993).

In the present study, we determined the effects of low TC-complex concentrations on the dynamics of individual microtubules at polymer mass steady state by video microscopy. We also correlated the actions of TC-complex on the individual dynamic parameters with the stoichiometry of TC-complex incorporation into the microtubules and with the changes induced by TC-complex in the polymer mass. We found that incorporation of TC-complex strongly reduced the rate and extent of growing and shortening and increased the percentage of time that the microtubules spent in an attenuated state, neither growing nor shortening detectably. At the lowest concentrations examined, TC-complex kinetically stabilized the microtubules without decreasing the polymer mass, the mean microtubule length, or the microtubule number concentration.

MATERIALS AND METHODS

Purification of Tubulin and Preparation of TC-Complex. Microtubule protein was isolated without glycerol by three cycles of polymerization and depolymerization; tubulin was purified from the microtubule protein by phosphocellulose chromatography as previously described (Toso et al., 1993). The tubulin was rapidly frozen as drops in liquid nitrogen and stored at −70 °C until ready for use. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as the standard. TC-complex was prepared by a modification of the method described by Vandecandelaere et al. (1994) by incubating 4 μM colchicine with 22 μM tubulin for 1 h at 36 °C. Under the conditions used, >80% of the colchicine was bound to tubulin. Fresh TC-complex was prepared for each experiment and used for no longer than 2 h after preparation to avoid possible aggregation or denaturation.

Video Microscopy. Tubulin pellets were thawed and centrifuged (48000*g*, 4 °C, 15 min) to remove any aggregated or denatured tubulin. Tubulin (1.5 mg/mL) was mixed with *Strongylocentrotus purpuratus* flagellar seeds in 87 mM Pipes, 36 mM Mes, 1.4 mM Mg^{2+}, 1 mM EGTA, and 1 mM GTP, pH 6.8 (PMMEG buffer), and incubated at 36 °C in the absence of TC-complex for 25 min to polymerize the microtubules (Panda et al., 1994). These buffer conditions yielded sufficiently rapid dynamics at steady state so that the suppressive actions of TC-complex on dynamics could be analyzed. The desired TC-complex concentration was then added to the microtubule suspension, and a new steady state was attained after an additional 30 min of incubation. Samples (2.5 μL) of microtubule suspensions were prepared for video microscopy, and the dynamics of individual microtubules at their plus ends were recorded at 36 °C as previously described (Panda et al., 1994). The dynamics were recorded for a maximum of 1 h. We used a computer-based analysis system (a gift from Dr. Neal Gliksman and

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[1] Abbreviations: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; PMMEG, 87 mM Pipes, 36 mM Mes, 1.4 mM Mg^{2+}, 1 mM EGTA, and 1 mM GTP, pH 6.8; TC-complex, final-state tubulin−colchicine complex; tubulin−GTP, tubulin−GDP, or tubulin−GDP•P_i, tubulin-liganded with GTP, GDP, or GDP•P_i at the exchangeable guanine nucleotide binding site.

Dr. E. D. Salmon, University of North Carolina, Chapel Hill) to analyze microtubule length changes with time. Length changes were measured in real time using a mouse-driven cursor, and data points were collected at 3–6 s intervals. Microtubules were measured until they underwent complete depolymerization to the axoneme seed or until the microtubule end became obscured. The length changes undergone by a particular microtubule as a function of time were then used to create a "life history" plot. The growing and shortening rates were determined by least-squares regression analysis of the data points for each growing or shortening phase. The reported mean growing and shortening rates represent the averaged values for all growing or shortening events observed for a particular reaction condition. We considered the microtubule to be in a growing phase if it increased in length by >0.2 μm at a rate >5 dimers/s and in a shortening phase if the microtubule shortened in length by >0.2 μm at a rate >5 dimers/s. Microtubule ends that changed ≤0.2 μm over the duration of 4–5 data points were considered to be in an attenuated state (*i.e.*, no detectable growing or shortening). A total of 15–25 microtubules were analyzed for each solution condition.

We calculated the catastrophe frequency (a catastrophe is a transition from the growing or attenuated state to shortening; Walker et al., 1988) by dividing the number of catastrophes by the sum of the total time spent in the growing plus attenuated states for all microtubules for a particular condition. The rescue frequency (a rescue is a transition from shortening to growing or attenuation, excluding new growth from a seed; Walker et al., 1988) was calculated by dividing the total number of rescue events by the total time spent shortening for all microtubules for a particular condition.

Microtubule Length Determinations. TC-complex was added to steady-state microtubule suspensions, and the mixtures were incubated for 70 min at 36 °C. Microtubules were then fixed with 0.25% glutaraldehyde and visualized and recorded immediately by video microscopy. Mean microtubule lengths were determined by measuring 200–250 microtubules at each TC-complex concentration.

Stoichiometry of TC-Complex Incorporation per Microtubule. TC-complex containing a trace amount of [^{3}H]-colchicine (specific activity, 1.03 Ci/mol) was added to steady-state microtubule suspensions and incubated for 70 min at 36 °C. Unbound ^{3}H-TC-complex was separated from the microtubules by centrifugation through 50% sucrose cushions for 70 min at 36 °C (100000*g*). Microtubule pellets were dissolved in PMMEG buffer containing 0.5 mM $CaCl_2$ at 0 °C, and the tubulin concentration in the pellets was determined (Bradford, 1976). The amount of ^{3}H-TC-complex incorporated in the microtubules was determined by scintillation spectrometry. The microtubule number concentration was determined from the polymer mass and mean length of microtubules for each TC-complex concentration using a value of 1690 tubulin dimers per micrometer of length. The number of TC-complexes incorporated per microtubule was determined by dividing the TC-complex concentration by the microtubule number concentration.

RESULTS

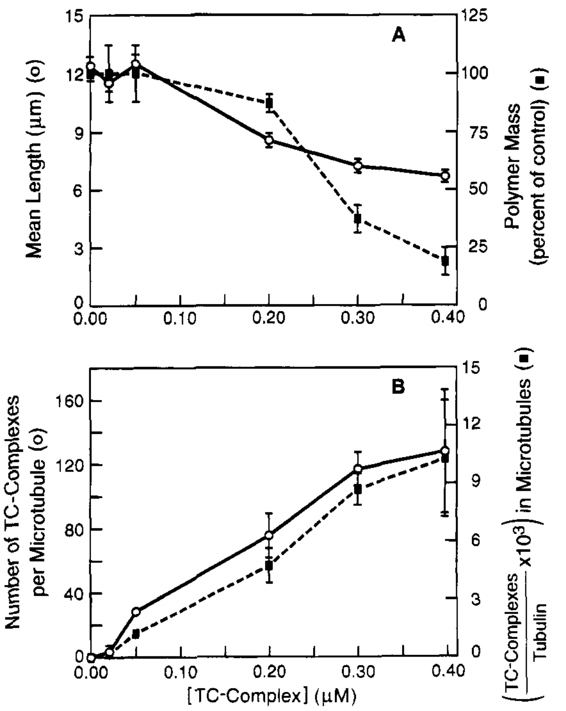

FIGURE 1: Effects of TC-complex on bulk phase microtubule assembly. Tubulin (1.5 mg/mL) was polymerized at the ends of axoneme seeds in PMMEG buffer at 36 °C for 25 min. Preformed TC-complex was added and incubation continued for an additional 70 min. (A) The effects of TC-complex on the polymer mass (squares) and on the mean microtubule length (open circles) were determined as described under Materials and Methods. (B) The stoichiometries of incorporated TC-complex per microtubule (open circles) and per mole of tubulin in microtubules (squares) were determined as described under Materials and Methods. Error bars = SEM.

Effects of TC-Complex on Bulk Phase Microtubule Assembly. Our goal was to understand how colchicine modulates microtubule polymerization dynamics at steady state. We used final-state TC-complex rather than free colchicine in this work to eliminate the time required for formation of the active form of the drug (Margolis & Wilson, 1977; Skoufias & Wilson, 1992). We first determined the microtubule polymer mass, the mean microtubule lengths, and the stoichiometry of incorporated TC-complex per microtubule after adding the complex and allowing the microtubules to reestablish steady state. Tubulin (1.5 mg/mL) was polymerized to polymer mass steady state at the ends of axonemal seeds at 36 °C. Then preformed TC-complex was added (at a range of concentrations between 0.02 and 0.4 μM complex), and the mixture was incubated for an additional 30 min. At high TC-complex concentrations (>0.05 μM, see below), the microtubules rapidly depolymerized, and a new steady state with fewer microtubules (depending upon the TC-complex concentration) was attained within approximately 30 min. The polymer mass and the mean lengths of the microtubules were then determined by measuring 200–250 microtubules at each TC-complex concentration (Materials and Methods); these are shown in Figure 1A.

TC-complex reduced the steady-state polymer mass in a concentration-dependent manner. Little or no depolymerization occurred at low TC-complex concentrations (between 0.02 and 0.05 μM). However, a significant reduction in the polymer mass occurred at higher concentrations (Figure 1A). Similarly, no change in the mean microtubule length occurred at or below 0.05 μM TC-complex (Figure 1A), but significant reduction in the mean lengths occurred above this concentration. For example, 0.4 µM TC-complex reduced the mean length by 46% (Figure 1A).

We also determined the amount of TC-complex incorporated into the microtubules 70 min after adding the complex (Figure 1B) (the extent of incorporation at a given TC-complex concentration increases with increasing time of incubation; Skoufias & Wilson, 1992). Incorporation of TC-complex increased with increasing TC-complex concentration. For example, at 0.02 µM TC-complex, 3 mol of TC-complex was incorporated per 10 000 mol of tubulin. At the highest TC-complex concentration used (0.4 µM), 100 mol of TC-complex was incorporated per 10 000 mol of tubulin in the microtubules. We also calculated the number of TC-complexes incorporated per microtubule; the stoichiometry varied from 3.7 TC-complexes per microtubule at 0.02 µM TC-complex to 128 per microtubule at 0.4 µM TC-complex (Figure 1B).

Effects of TC-Complex on Microtubule Dynamics. We analyzed microtubule dynamics at steady state after TC-complex-induced depolymerization, if it occurred, was complete. Under the experimental conditions used, microtubule growth occurred predominantly at one end of the seeds and was considered to be the plus ends as previously described (Walker et al., 1988; Toso et al., 1993; Panda et al., 1994). The steady-state microtubules displayed length changes (Figure 2A) typical of those observed in earlier reports (Walker et al., 1988; Gildersleeve et al., 1992; Toso et al., 1993; Panda et al., 1994; Derry et al., 1995). TC-complex concentrations as low as 0.05 µM suppressed dynamics (Figure 2B). For example, it is apparent from visual inspection of the life-history plots that 0.05 µM TC-complex increased the rescue frequency and increased the percentage of time the microtubules spent in the attenuated state. The microtubules were clearly very strongly stabilized at 0.4 µM TC-complex (Figure 2C).

TC-Complex Reduced the Rates and Extents of Shortening and Growing. The quantitative effects of TC-complex on the individual dynamic instability parameters obtained by measuring 15−25 microtubules at each concentration are summarized in Table 1. In the absence of TC-complex, the mean growing rate was 38 dimers/s; TC-complex (0.02−0.4 µM) strongly reduced the growing rate in a concentration-dependent manner. TC-complex also strongly reduced the mean shortening rate, and the suppression of the shortening rate by TC-complex was actually stronger than the suppression of the growing rate at low TC-complex concentrations. For example, 0.02 µM TC-complex suppressed the shortening rate by ~24% whereas it suppressed the growing rate by only ~12%.

In the low TC-complex concentration range (0.02−0.05 µM), suppression of the shortening and growing rates occurred in the absence of detectable changes in the microtubule polymer mass or in the mean microtubule length. For example, 0.05 µM TC-complex reduced the shortening and growing rates by 47% and 34%, respectively, while the microtubule lengths and the polymer mass were not detectably affected (Table 1, Figure 1A). Thus, microtubule growing and shortening dynamics appear to be considerably more sensitive to low concentrations of TC-complex than bulk phase polymerization. Because these actions of low TC-complex concentration occurred in the absence of changes in the soluble tubulin concentration, the data also confirm that TC-complex acts on the microtubule ends.

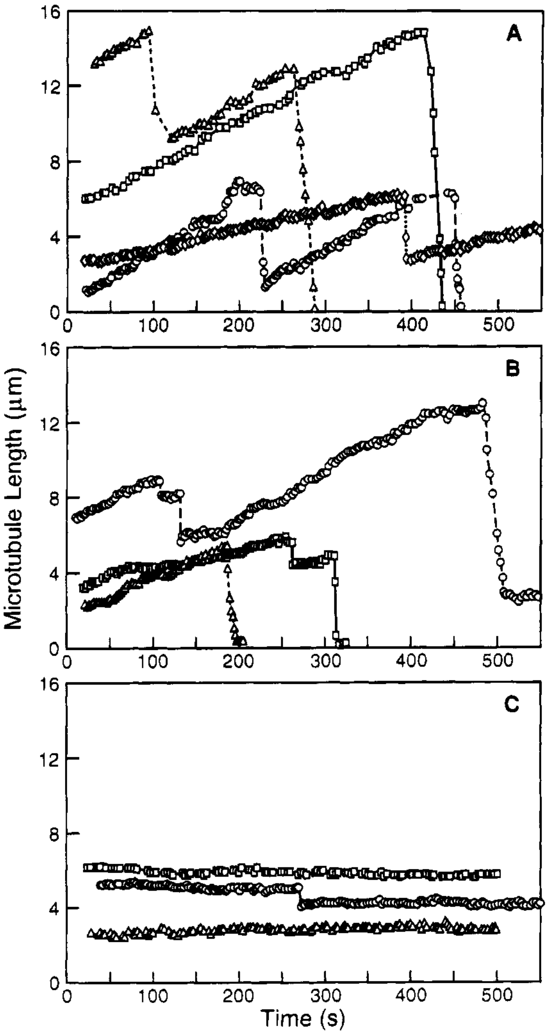

FIGURE 2: Life-history plots of microtubules at steady state in the absence (A) and presence of 0.05 µM (B) and 0.4 µM (C) TC-complex. The lengths of individual microtubules were measured from real time video tape recordings as described under Materials and Methods.

We also determined the mean microtubule length shortened per shortening event by dividing the summed shortening lengths for all microtubules for a particular condition by the total number of shortening events. As shown in Figure 3, TC-complex strongly reduced the lengths that microtubules shortened during the shortening events. For example, 0.4 µM TC-complex reduced the mean length shortened per shortening event by a factor of 2.6 from 6.8 µm in controls to 2.6 µm. Similarly, the length grown per growing event decreased with increasing TC-complex concentrations (Figure 3).

TC-Complex Decreased the Percentage of Time the Microtubules Spent Growing and Shortening and Increased the Percentage of Time Microtubules Spent in the Attenuated State. TC-complex reduced the percentage of time the microtubules spent growing in a concentration-dependent manner (Table 1). For example, 0.05 µM TC-complex reduced the percentage of time growing from 85.1% to 70.5%. At 0.4 µM TC-complex, the highest concentration studied, the percentage of time growing was dramatically reduced, to 8.8%. At low TC-complex concentrations (0.02−0.05 µM), there was no detectable effect on the percentage of time the microtubules spent shortening.

Table 1: Actions of TC-Complex on Microtubule Dynamics at Steady State

	TC-complex concentration (μM)					
	0	0.02	0.05	0.2	0.3	0.4
			Rates (Dimer/s)			
growing	38.3 ± 10.0[a]	33.6 ± 12.0	25.5 ± 12.0	19.3 ± 12.0	8.7 ± 2.7	9.1 ± 3.2
shortening	1030 ± 310	781 ± 210	547 ± 310	445 ± 440	216 ± 500	189 ± 230
			Percent of Total Time			
growing	85.1	87.5	70.5	44.2	7.6	8.8
shortening	9.3	10.6	11.7	7.9	4.2	5.9
attenuation	5.6	1.9	17.8	47.9	88.2	85.4

[a] ± = standard deviation.

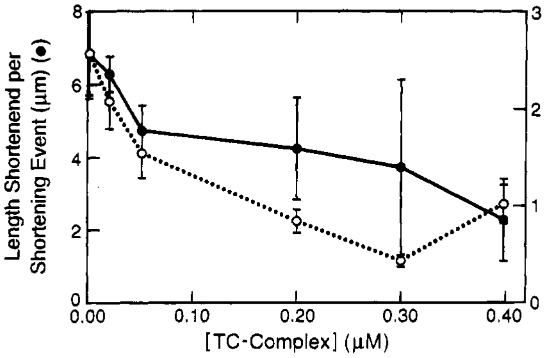

FIGURE 3: Microtubule length changes per growing (open circles) or shortening (closed circles) event as a function of TC-complex concentration. The mean lengths the microtubules shortened during shortening events were calculated by dividing the summed shortening lengths for all microtubules for a particular condition by the total number of shortening events measured for that condition. The growing lengths per growing event were calculated similarly. Error bars = SEM.

However, higher concentrations of TC-complex substantially reduced the percentage of time the microtubules spent shortening.

Both in cells and *in vitro* at steady state, microtubules spend a significant fraction of time in an attenuated (apparent pause) state, neither growing nor shortening detectably (Toso et al., 1993; Panda et al., 1994; Shelden & Wadsworth, 1993; Dhamordharan & Wadsworth, 1995). Subunit addition and loss may be occurring when microtubules are in an attenuated state, but the extent of net growing or shortening is too low to be detected by video microscopy. As shown in Table 1, TC-complex strongly increased the percentage of time that the microtubules spent in the attenuated state. At 0.4 μM TC-complex, the percentage of time the microtubules spent in an attenuated state increased 16-fold from 5.5% to 85%. Such a strong increase reflects a virtual elimination of detectable growing and shortening events.

Effects of TC-Complex on the Transition Frequencies. The catastrophe and rescue frequencies are important parameters that reflect the loss and gain of the stabilizing GTP or GDP•P$_i$ cap at microtubule ends. These transitions are thought to play a key role in the regulation of dynamic instability and microtubule function in cells (Belmont et al., 1990; Gliksman et al., 1993). The transition from growing or attenuation to shortening is called catastrophe, and the transition from shortening to growing or attenuation is called rescue (Walker et al., 1988). TC-complex decreased the catastrophe frequency and increased the rescue frequency in a concentration-dependent manner as shown in Figure 4. For example, 0.4 μM TC-complex reduced the catastrophe frequency 7-fold and increased the rescue frequency 4-fold. Even at very low concentrations, *e.g.*, 0.05 μM, TC-complex significantly decreased the catastrophe frequency (1.3-fold) and increased the rescue frequency (2-fold). The stoichiometry of incorporated TC-complex is extremely low at low TC-complex concentrations (~28 TC-complexes per microtubule after 70 min of incubation with 0.05 μM TC-complex). Thus, incorporation of low numbers of TC-complexes per microtubule significantly modulates the transition frequencies, indicating that incorporation of low numbers of TC-complex at microtubule ends may directly modulate the GTP or GDP•P$_i$ cap mechanism (see Discussion).

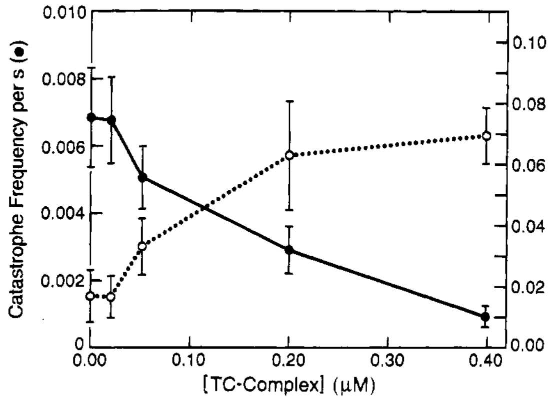

FIGURE 4: Catastrophe (closed circles) and rescue (open circles) frequencies as a function of TC-complex concentration. Catastrophe and rescue frequencies were calculated as described under Materials and Methods. Error bars = SD.

Effects of TC-Complex on Dynamicity. We define dynamicity as the total detectable tubulin dimer exchange per second. Dynamicity is calculated from all detectable growing and shortening events including the time spent in the attenuated state (Toso et al., 1993; Panda et al., 1994). As shown in Figure 5, TC-complex reduced the dynamicity in a concentration-dependent manner. Dynamicity was almost completely inhibited at high TC-complex concentrations (≥0.3 μM TC-complex) under conditions in which ~100 or more TC-complexes were incorporated per microtubule. Dynamicity was strongly reduced even at TC-complex concentrations that did not reduce the steady-state microtubule polymer mass or the mean length of the microtubules. For example, 0.05 μM TC-complex suppressed dynamicity by 36% (Figure 5) while it had no detectable effect either on the polymer mass or on the mean microtubule length (Figure 1A). These actions of low TC-complex concentrations occurred at very low stoichiometries of incorporated TC-complex in the microtubules. Thus, they cannot be due to extensive copolymerization of TC-complex into the microtubules, but, rather, they must be due to the presence of small numbers of TC-complexes at the microtubule ends.

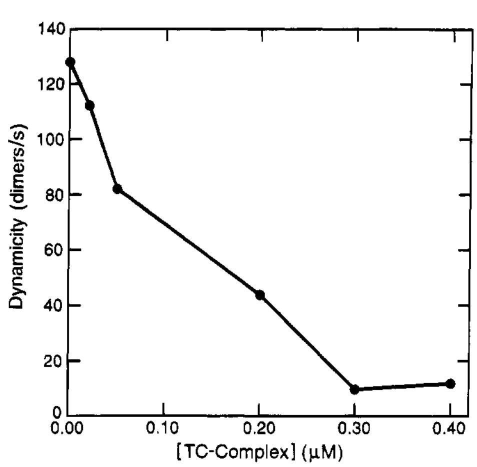

FIGURE 5: Action of TC-complex on microtubule dynamicity. Dynamicity was calculated from all detectable growing and shortening events including the time that the microtubules spent in the attenuated state.

DISCUSSION

The experiments reported here demonstrate that incorporation of low numbers of TC-complexes into microtubules at steady state *in vitro* strongly stabilizes microtubules. TC-complex strongly suppressed the rates and extents of growing and shortening. In addition, TC-complex strongly reduced the catastrophe frequency, and it increased the rescue frequency. Microtubules *in vitro* (Toso et al., 1993; Panda et al., 1994) and in living cells (Sammak & Borisy, 1988; Shelden & Wadsworth, 1993) spend a significant fraction of their total time in a metastable or attenuated state, neither growing nor shortening detectably, and one of the most dramatic actions of TC-complex was to strongly increase the percentage of time that the microtubules spent in the attenuated state (Figure 2, Table 1). Specifically, at 0.4 μM TC-complex, the highest TC-complex concentration examined, the percentage of time microtubules spent in the attenuated state increased 16-fold from 5.5% to 85.4%.

Mechanism of Kinetic Stabilization of Microtubules by TC-Complex. Suppression of Growing Rates by TC-Complex. TC-complex strongly reduced the mean growing rates of individual steady-state microtubules in a concentration-dependent manner. Similar results were also obtained by Vandecandelaere et al. (1994). In addition, low TC-complex concentrations (0.02 and 0.05 μM) reduced growing rates in the absence of detectable changes in the polymer mass. For example, 0.05 μM TC-complex reduced the growing rate by ~50% while it did not detectably affect the soluble tubulin concentration. Microtubule growth rate is represented by the equation:

$$R_g = K_+C - K_- \qquad (1)$$

where R_g is the growing rate, K_+ is the association rate constant, K_- is the dissociation rate constant, and C is the soluble tubulin concentration. Because C was not affected by low TC-complex concentrations, the reduction in the growing rate could only be due to reduction of K_+ or to an increase in K_-. Because TC-complex strongly reduced the shortening rate, it is expected that TC-complex does not increase K_-. [TC-complex also decreases the rate of shortening upon dilution-induced disassembly of microtubules (Margolis et al., 1980; Farrell & Wilson, 1984; Bergen & Borisy, 1986), further supporting the idea that TC-complex does not increase K_-.] Thus, we can conclude that TC-complex decreases the association rate constant for tubulin addition at the microtubule plus ends.

Inhibition of tubulin addition at the microtubule ends by incorporated molecules of TC-complex could occur by steric hindrance due to the presence of the more bulky TC-complexes at the ends as compared with tubulin. Another possibility is that colchicine might alter the structure of the microtubule end in such a way that further incorporation of tubulin becomes energetically unfavorable. The structural perturbation induced by TC-complex at microtubule ends may be due to a changed tubulin conformation and/or to restricted flexibility of tubulin when bound to colchicine. Colchicine increases the association constant for the α–β tubulin binding in the dimer, and, therefore, it may increase subunit contacts in dimeric tubulin (Detrich et al., 1982; Panda et al., 1992).

Soluble tubulin has a very low intrinsic GTPase activity, but binding of colchicine to tubulin strongly stimulates the GTP hydrolysis rate (David-Pfeuty et al., 1979; Perez-Ramirez & Timasheff, 1994). Thus, binding of colchicine to tubulin appears to induce a conformational change in the tubulin that may partly mimic the conformation of polymerized tubulin. It seems reasonable to postulate that TC-complex has an altered flexibility and conformation so that when incorporated at the microtubule end it is unable to adopt a binding geometry that is necessary for the efficient addition of further tubulin–GTP. Newly added tubulin might assume an incorrect geometry that makes future tubulin addition energetically unfavorable in a propagated manner. Such a mechanism can explain why TC-complex reduces the growing rate. Microtubules might resume normal growth rates if the TC-complex dissociates or if it is buried by addition of a sufficient number of new tubulin dimers so that the original defect becomes neutralized. Although energetically unfavorable, tubulin continuously adds to microtubule ends along with low numbers of TC-complex, albeit at a much lower rate than in the absence of TC-complex (Sternlicht & Ringel, 1979; Farrell & Wilson, 1984). The extent of TC-complex incorporation at the microtubule ends appears to determine the degree of microtubule stabilization, but the continued incorporation of TC-complex into the microtubule core does not appear to be the primary mechanism responsible for the ability of TC-complex to suppress microtubule dynamics.

Suppression of Shortening Rates by TC-Complex. TC-complex strongly inhibited the steady-state shortening rate (Table 1). For example, 0.05 μM TC-complex reduced the shortening rate by approximately 50% (Table 1) while there was no detectable change in the soluble tubulin concentration and polymer mass (Figure 1A). Thus, the results demonstrate unambiguously that TC-complex stabilizes microtubule ends.

Interestingly, the shortening rate was inhibited more strongly than the growing rate at low TC-complex concentrations. These results may explain why low concentrations of colchicine do not induce significant disassembly when added to preformed microtubules. The conformational changes in tubulin due to the binding of colchicine may change the geometry of tubulin controlling the lateral interactions between tubulin subunits in adjacent protofilaments, thus strengthening the interactions between the protofilaments. Evidence in support of this idea was previously obtained by Andreu et al. (1983).

Microtubules are thought to be stabilized at their ends when the terminal subunits are composed of tubulin−GTP (tubulin bound to GTP at the exchangeable site) or of tubulin−GDP•P_i and become highly unstable and rapidly depolymerize when the stabilizing cap is lost, thus exposing tubulin−GDP at the microtubule ends (Mitchison & Kirschner, 1984; Carlier, 1989; Stewart et al., 1990; Erickson & O'Brien, 1992). The strength of the tubulin−tubulin interactions in the microtubule lattice appears to be regulated by the hydrolysis of GTP and release of P_i at or shortly after tubulin−GTP adds to the microtubule ends (Erickson & O'Brien, 1992; Hyman et al., 1995). The stabilizing action of TC-complex at microtubule ends may be due to strengthening of the stabilizing tubulin−GTP or tubulin−GDP•P_i cap. It is possible that TC-complex dissociates from microtubule ends much more slowly than tubulin−GDP because it induces a conformational change in tubulin that mimics the conformation of the stabilizing tubulin−GTP or tubulin-GDP•P_i cap or because it assumes an entirely different stabilizing conformation.

Effects of TC-Complex on the Transition Frequencies. Catastrophe (transition to shortening) may occur upon loss of the last molecule of tubulin−GDP•P_i or tubulin−GTP, and rescue may be initiated by binding of one or a few tubulin−GTP molecules in a favorable region at the end of a rapidly shortening microtubule. Low numbers of incorporated TC-complexes at the microtubule ends strongly reduced the catastrophe frequency (Figures 1, 4). TC-complex may have reduced the catastrophe frequency either by lowering the rate of P_i release after GTP hydrolysis and/or by lowering the rate of TC-complex−GDP dissociation as compared to the dissociation rate for tubulin−GDP. Such actions could be due to the conformational changes induced in tubulin by the binding of colchicine. Thus, it is possible that TC-complex is directly acting on the stabilizing GTP or GDP•P_i cap. Low numbers of incorporated TC-complex also strongly increased the rescue frequency (Figures 1, 4) which indicates that the stabilizing cap is rapidly regained. Because TC-complex strongly suppresses the shortening rate, regain of the stabilizing cap may occur indirectly because the microtubule has sufficient time during a shortening event to become recapped before undergoing complete depolymerization.

TC-Complex Decreases Polymer Mass yet Kinetically Stabilizes Microtubule Dynamics. In the present study, the microtubules were strongly stabilized by TC-complex regardless of the extent to which the polymer mass decreased. For example, 0.2 μM TC-complex decreased the polymer mass by ~20%, and yet it decreased the dynamicity of the microtubules by 50% and increased the percentage of time that the microtubules spent in the attenuated state by 8-fold. It may seem paradoxical that microtubules depolymerize upon addition of high concentrations of TC-complex or colchicine, yet the surviving microtubules are highly stabilized. This apparent paradox can be explained by making a distinction between the steady-state polymer mass and critical subunit concentration, which are thermodynamic parameters, and microtubule growing and shortening dynamics, which are kinetic parameters. Colchicine binds strongly to soluble tubulin with a free energy of ~−9 kcal/mol at 37 °C (Bhattacharyya & Wolff, 1974), but microtubules have very low affinity for free colchicine (Wilson & Meza, 1973; Lee et al., 1974; Skoufias & Wilson, 1992). Linked-function theory (Wyman, 1964; Wyman & Gill, 1990) indicates that the presence of ligands in a polymerization reaction shifts the equilibrium toward the species of greater binding. Thus, the presence of colchicine will be expected to shift the polymerization equilibrium toward soluble tubulin because colchicine binds soluble tubulin more strongly than microtubules. When colchicine binds to tubulin, the tubulin undergoes a conformational change and the TC-complex has a lower free energy than that of free tubulin. Thus, the presence of TC-complex also increases the free energy of polymerization (Sternlicht et al., 1980, see Table IV). An additional increase in the free energy for polymerization may result from perturbation of the microtubule end by the incorporated TC-complex. Thus, when a sufficiently high concentration of TC-complex is added to a suspension of steady-state microtubules, the polymerization equilibrium will shift toward soluble tubulin and the microtubules will depolymerize until a new steady state at a higher critical concentration is reached, a thermodynamic requirement of the system. Such transitions to a new steady state at lower polymer mass, which could involve substantial microtubule depolymerization at high colchicine or TC-complex concentrations, do not indicate that the microtubules are kinetically destabilized.

Implications for Microtubule-Based Cell Function. Mitotic spindle microtubules are highly dynamic, and recent evidence indicates that the rapid dynamics of spindle microtubules, not just their presence in spindles, are very important for proper spindle function (McIntosh & Hering, 1991; Jordan et al., 1991, 1992, 1993). For example, in prometaphase of mitosis, microtubules originating at the centrosomes appear to probe the cytoplasm by rapid growing and shortening until kinetochore attachment is achieved (Rieder & Alexander, 1990; Hayden et al., 1990). Similarly, tension on kinetochores which may be produced by dynamic microtubules appears to be important in signaling the transition from metaphase to anaphase (Li & Nicklas, 1995). Also, Lombillo et al. (1995 a,b) have recently obtained data indicating that rapid depolymerization of kinetochore microtubules may act together with motor molecules to effect chromosome movements during anaphase.

Colchicine is a well-studied inhibitor of mitosis that has been believed to act by disrupting the microtubules required for chromosome movement during mitosis. Recent evidence has indicated that two other antimitotic drugs, vinblastine and taxol, inhibit mitosis by suppressing the dynamics of spindle microtubules rather than by depolymerizing the microtubules (Jordan et al., 1991, 1992, 1993). On the basis of the similar ability of these drugs and colchicine to stabilize microtubules at low concentrations *in vitro* (see below), it seems reasonable to suggest that colchicine may also inhibit mitosis at its lowest effective concentrations at least in part by stabilizing spindle microtubule dynamics rather than by depolymerizing the microtubules. In the present study, we found that the most potent action of TC-complex was suppression of the plus end shortening rate. Specifically, a very low concentration of TC-complex (0.05 μM) suppressed the shortening rate by 50%. Thus, one possibility is that TC-complex may restrict depolymerization-dependent chromosome movement by suppressing plus end microtubule shortening.

Colchicine and vinblastine suppress microtubule polymerization by different molecular mechanisms. Specifically, vinblastine and colchicine bind to different regions of tubulin (Wilson & Jordan, 1994), and at low concentrations, vinblastine appears to bind directly to microtubule ends (Wilson et al., 1982) while colchicine must first form a final-state TC-complex which then adds to the ends (Margolis & Wilson, 1977; Skoufias & Wilson, 1992). In addition, vinblastine binding to tubulin is rapid and reversible, whereas the binding of colchicine to tubulin is slow and poorly reversible (Wilson & Jordan, 1994; Hamel, 1990). Also, TC-complex can form microtubule copolymers but vinblastine cannot (Jordan & Wilson, 1990). Another important antimitotic drug that acts on microtubules is taxol. Taxol does not appear to bind soluble tubulin (Schiff et al., 1979; Diaz & Andreu, 1993), but rather, it acts by binding to tubulin in microtubules. Taxol can bind to microtubules with high stoichiometry by binding along the microtubule surface (Schiff et al., 1979; Diaz & Andreu, 1993; Derry et al., 1995). The action of taxol is very different from that of colchicine and vinblastine. Taxol reduces the critical subunit concentration for polymerization, and it strongly stimulates microtubule polymerization while colchicine and vinblastine inhibit microtubule polymerization (Wilson & Jordan, 1994). A number of significant qualitative differences also exist in the actions of colchicine, vinblastine, and taxol on microtubule dynamics. For example, TC-complex and taxol reduce the shortening rate more strongly than the growing rate (Table 1) whereas vinblastine inhibits both the growing and the shortening rate to similar extents (Toso et al., 1993; Derry et al., 1995). Both vinblastine and colchicine appear to suppress microtubule dynamics by binding at the microtubule ends, most probably by altering the nature and/or size of the stabilizing cap, whereas substoichiometric concentrations of taxol kinetically stabilize microtubule dynamics by binding along the length of the microtubules apparently without directly altering the cap (Carlier & Pantaloni, 1983; Wilson et al., 1985; Derry et al., 1995).

Despite the differences in the nature of the molecular interactions of vinblastine, taxol, and colchicine with tubulin and microtubules and the differences in their abilities to modulate the polymer mass at high drug concentrations, all three drugs stabilize microtubule dynamics at low concentrations. For example, colchicine, vinblastine, and taxol all reduce the rate and extent of microtubule shortening and growing at steady state and they increase the percentage of time that microtubules spend in the attenuated state (Toso et al., 1993; Derry et al., 1995). Thus, a number of molecules stabilize microtubule dynamics by interacting with microtubule surfaces and ends by different molecular mechanisms. The powerful ability of colchicine and other naturally-occurring compounds to stabilize microtubule dynamics strongly supports the idea that cells have natural regulatory molecules that stabilize microtubule dynamics. Based upon the apparent diversity of the molecular mechanisms of such drugs, it seems that cells may have evolved a number of different mechanisms for regulating microtubule stability.

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