

Autoregulation of Tubulin Synthesis in Hepatocytes and Fibroblasts

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ABSTRACT Microtubule polymer levels in mouse 3T6 fibroblasts and primary cultures of rat hepatocytes can be manipulated by treatment of cells with long term, low doses of colcemid. Such treatment produces a rather uniform population of cells with microtubules of reduced lengths. Using this system, we demonstrate (a) that the rate of tubulin synthesis is sensitive to small changes (10%) in microtubule polymer mass and (b) that the percent of inhibition of synthesis is proportional to the level of soluble tubulin. Experiments with hepatocytes indicate that not only synthesis but the stability of tubulin protein was also regulated to maintain a specific level of tubulin. Treatment of hepatocytes with colcemid or other microtubule-depolymerizing drugs reduced the half-life of tubulin from 50 to 2 h, whereas taxol, which stabilizes microtubules, increased the half-life. To assess the consequences of altering microtubule polymer mass, we have analyzed the effect of controlled depolymerization of microtubules in rat hepatocytes on the processing of endocytosed ligands and found it sensitive to small changes in microtubule polymer levels.

Cytoplasmic microtubules play a role in such cellular processes as secretion and intracellular transport (19, 39, 41, 45), maintenance of cell shape (11, 34, 51), and directional cell movement (1, 52). Since microtubules are in dynamic equilibrium with their subunits, normal cell function may require the maintenance of a specific microtubule polymer/soluble tubulin ratio. Changes in this ratio have been associated with specific cellular activities (4, 24, 31, 37, 38). A variety of mammalian cells are capable of regulating the soluble tubulin concentration by controlling the rate of tubulin synthesis. For example, Ben-Ze'ev et al. (3) have demonstrated that treatment of cells with colchicine to depolymerize microtubules decreased the rate of tubulin synthesis. These studies were confirmed and extended by Cleveland et al. (12) who demonstrated that the amount of tubulin mRNA decreased in response to the elevation of tubulin monomer levels. Cleveland et al. (13) have further argued that tubulin monomer is the effective species since microinjection of sufficient tubulin into Chinese hamster ovary cells to increase cellular tubulin levels by ~50% also suppressed tubulin synthesis. This eliminated the possibility that inhibition of tubulin synthesis was due to a nonspecific effect of the depolymerizing drugs.

Although these results indicated that an autoregulatory mechanism exists whereby tubulin synthesis is responsive to

changes in the level of nonpolymerized tubulin, all of these studies involved massive perturbation of the microtubule polymer/monomer equilibrium which is not expected to be encountered in vivo. In particular, complete disassembly induced by drugs or injection of large amounts of soluble tubulin could lead to a stress response of the cell which would not be typical of normal cellular processes. Since most evidence suggests that cytoplasmic microtubules undergo subtle changes in vivo, we sought to determine how sensitive tubulin synthesis is to small perturbations in microtubule polymer/soluble tubulin levels.

We have studied in detail the relationship between tubulin synthesis and polymer levels, as measured biochemically, in response to low levels of microtubule-depolymerizing drugs, and we have found that synthesis is sensitive to even small changes in polymer mass. By comparing two cell types which contain different ratios of polymerized and nonpolymerized tubulin, mouse 3T6 fibroblasts and primary cultures of rat hepatocytes, we found that the magnitude of inhibition of synthesis was proportional to changes in the level of soluble tubulin. Thus, a feedback system exists in cells to respond to even slight perturbations in the level of nonpolymerized tubulin.

Small changes in the microtubule polymer/soluble tubulin

ratio can have other important physiological consequences. As with regulation of tubulin synthesis, the processing of endocytosed ligands in rat hepatocytes was found to be sensitive to small changes in microtubule polymer mass. These results suggest that cells can respond to small variations in microtubule polymer/soluble tubulin levels by regulating tubulin synthesis which, in turn, may have important consequences for microtubule-dependent functions.

MATERIALS AND METHODS

Cell Cultures

An established cell line of mouse 3T6 fibroblasts was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were used when 70–80% confluent. Rat hepatocytes in nonproliferating monolayer culture were obtained from the University of California, San Francisco, Liver Core Center Facility under the direction of Dr. D. M. Bissell. Hepatocytes were isolated to 99% purity from livers of adult male Sprague-Dawley rats by a collagenase perfusion system previously described (7), and then plated on collagen-coated plastic petri dishes in L-199 culture medium supplemented with glucose, insulin, corticosterone, rat serum, and penicillin G. After 24 h in culture, the medium was replaced with L-199 culture medium supplemented with only glucose and penicillin G; i.e., depleted medium. Use of depleted medium was necessary for studies on the effect of hormones of tubulin synthesis (Caron, J. M., manuscript in preparation). For consistency we used the same conditions for pharmacological experiments described here. Cell viability, as judged by granularity of the cytoplasm, cell shape, and attachment to substratum, required the presence of serum and hormones only during the first 24 h in culture. After this period, cells remained viable in depleted medium for at least 72 h. Hepatocytes were routinely used after 48 h in culture.

Preparation of Antibodies

Microtubule protein was prepared from bovine brain by the method of Shelanski et al. (44) with modifications described by Weingarten et al. (47). Tubulin was purified by chromatography on phosphocellulose (48). One-dimensional gel electrophoresis (28) was used to assess the purity of tubulin. Antiserum to tubulin was produced in female New Zealand white rabbits and monospecific anti-tubulin antibody was purified from whole sera by affinity chromatography as described by Cleveland et al. (12). This polyclonal antibody was specific for β -tubulin protein.

Lyophilized fluorescein-conjugated and unlabeled goat anti-rabbit antibodies were purchased from Cappel Laboratories, Inc., Cochranville, PA.

Immunofluorescence

Rat hepatocytes were plated on collagen-coated glass coverslips while ethanol-cleaned glass coverslips were used for fibroblasts. Cells were rinsed in phosphate-buffered saline (PBS) at 37°C followed by a 10-min, 37°C incubation in a fixation buffer which contained the following: 0.1 M PIPES, pH 6.75, 4% polyethylene glycol (PEG)-6000,¹ 1 mM EGTA, 1 mM MgSO₄, 1% Triton X-100, 2% formaldehyde. Cells were transferred to -20°C methanol for 5 min. All subsequent steps were performed at room temperature. After two consecutive 5-min washes in PBS, 0.1% Triton X-100, cells were incubated with anti-tubulin antibody diluted 1:10 in PBS for 30 min. After three 5-min washes in PBS, 0.1% Triton X-100, cells were incubated for 30 min with fluorescein-labeled goat anti-rabbit antibody diluted 1:100 in PBS followed again by three washes. Coverslips were mounted on glass slides in 90% glycerol, 0.1 M NaHCO₃, and photographed using a Zeiss photomicroscope III. Kodak Tri-X films were developed in D19.

Microtubule Polymer

IMMUNOBLOT ANALYSIS: Cells were plated in 35-mm plastic petri dishes. All extraction steps were performed at 37°C. After one rinse in PBS, cells were washed once in microtubule-stabilizing buffer (MSB) (0.1 M PIPES, pH 6.75, 1 mM EGTA, 1 mM MgSO₄, 2 M glycerol or 4% PEG-6000, and protease inhibitors [10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 0.5 mM benzamide, 5 μ g/ml pepstatin A, 5 μ g/ml O-phenanthroline, 0.5 mM phenylme-

thylsulfonyl fluoride]), and extracted with 0.5 ml MSB containing 0.1% Triton X-100. After 15 min, this solution was gently removed and replaced with 0.5 ml fresh MSB plus 0.1% Triton X-100 for an additional 15 min. The final extraction solution was removed and 300 μ l of lysis buffer (25 mM Tris-HCl, pH 7.4, 0.4 M NaCl, 0.5% SDS) was added to solubilize cytoskeletal proteins remaining on the plates. After 5 min at 37°C, cell lysates were transferred to 1.5-ml microfuge tubes, boiled for 3 min, and centrifuged at 12,000 g for 2 min. Supernatants containing solubilized cytoskeletal proteins were transferred to another 1.5-ml tube and β -mercaptoethanol was added to 0.1%. The sample was boiled for 3 min and either kept on ice for immediate analysis or stored at -70°C for up to 2 wk.

Equal amounts of protein (7 μ g) were electrophoresed in 8.5% SDS polyacrylamide gels (0.7 x 16.5 x 12 mm) according to the method of Laemmli (28), except that the pH of the running gel was adjusted to pH 9.1. After electrophoresis, proteins were transferred to nitrocellulose (Schleicher & Schuell, Inc., Keene, NH) using a Hoefer electroblot apparatus (Hoefer Scientific Instruments, San Francisco, CA). Nitrocellulose blots were incubated with anti-tubulin antibody followed by incubation with 1×10^6 cpm/ml ¹²⁵I-labeled goat anti-rabbit antibody as described by Burnette (10). The chloramine T method was used to label the secondary antibody (16). Carrier-free ¹²⁵I was purchased from Amersham Corp., Arlington Heights, IL. Nitrocellulose blots were autoradiographed at -70°C using Kodak X-omat AR x-ray film and Dupont Cronex lighting plus intensifying screens. Blots were exposed for variable lengths of time to insure that autoradiographs gave a linear response within the range of tubulin concentrations found in polymer fractions after controlled depolymerization. After autoradiography, protein patterns on nitrocellulose blots were visualized by staining with 0.1% Amido black, 50% methanol, 10% acetic acid, followed by destaining with 80% methanol, 5% acetic acid.

IMMUNOPRECIPITATION: Cells were plated in 35-mm dishes. After 24 h, the medium was changed and [³⁵S]methionine (Amersham Corp.; 50 μ Ci/ml) was added for an additional 24 h. Cells were extracted to separate microtubule polymer from soluble tubulin as described above. Lysis buffer was adjusted to optimize conditions for immunoprecipitation (0.25 M Tris-HCl, pH 7.4, 0.4 M NaCl, 0.1% deoxycholate, 1% Nonidet P-40, 0.5% SDS). Equal amounts of trichloroacetic acid-precipitable counts were used in the immunoprecipitation assay. Control experiments demonstrated antibody excess in this assay. Immunoprecipitation of tubulin protein was performed as described by Cleveland et al. (12) with modifications suggested by Firestone et al. (15). Immunoprecipitates were analyzed by one-dimensional gel electrophoresis as described above. Gels were fluorographed according to the method of Bonner and Laskey (9), dried and autoradiographed using Kodak X-omat AR x-ray film at -70°C. We note that the method of quantitating microtubule polymer levels by long-term labeling and immunoprecipitation is applicable only if experimental conditions do not significantly alter the specific activity of tubulin protein during the course of the experiment.

Percent Polymer and Soluble Tubulin

Percent polymer was determined by comparing the amount of tubulin protein remaining in extracted cytoskeletons with total cellular extracts. Cytoskeletons were prepared from monolayers of cells plated in 35-mm dishes as described above and finally solubilized in 300 μ l of the appropriate lysis buffer for either immunoblot analysis or immunoprecipitation. To prepare total cellular extracts, cells plated in 35-mm dishes were washed once in PBS, once in MSB, and lysed with 300 μ l of lysis buffer. Equal volumes of whole cell and polymer extracts were analyzed. Although detachment of cells during washing and extraction was closely monitored and did not present an obvious problem, error due to unequal loss of cells was minimized by using plates in triplicate and averaging the results. Percent soluble tubulin was calculated from percent polymer.

Tubulin Synthesis

Cells were plated in 16-mm wells. Newly synthesized proteins were labeled for 30 min with [³⁵S]methionine (Amersham Corp.; 100 μ Ci/150 μ l), cells were lysed, and immunoprecipitation of tubulin protein was performed as described above.

Endocytosis Assay

Asialo-orosomucoid (ASOR) was kindly provided by Dr. R. D. Klausner (National Institutes of Health). The glycoprotein was iodinated with ¹²⁵I (Amersham Corp.; carrier-free) by the chloramine T method (16). The degradation assay was carried out essentially as described by Wolkoff et al. (50) except that incubation of cells with ¹²⁵I-ASOR was performed at 37°C for 10 min rather than 4°C for 60 min to maintain microtubule integrity. Briefly, hepatocytes

¹ Abbreviations used in this paper: ASOR, asialo-orosomucoid; MSB, microtubule-stabilizing buffer; PEG, polyethylene glycol.

were plated in 35-mm dishes. After 48 h in culture, cells were washed twice in depleted medium and incubated with a range of colcemid concentrations for 3 h. These and all subsequent steps were carried out at 37°C. Cells were washed three times in depleted medium \pm colcemid, incubated with 3 $\mu\text{g}/\text{ml}$ ^{125}I -ASOR for 10 min, washed twice in depleted medium \pm colcemid, one time in depleted medium containing 0.5 mM *N*-acetyl-D-galactosamine, one last time with depleted medium \pm colcemid, and finally recovered with 1.5 ml of depleted medium \pm colcemid. At time points from 0–3 h, degradation of ^{125}I -ASOR was assayed by removing 0.25 ml of the medium covering cells and adding this aliquot to an equal volume of ice-cold 10% trichloroacetic acid, 4% phosphotungstic acid. The sample was vortexed, kept on ice for at least 10 min, centrifuged for 10 min at 12,000 g, and an aliquot (200 μl) of the supernate was diluted into 10 ml Aquasol (New England Nuclear, Boston, MA) and counted in a Packard Tri-carb scintillation counter. After removal of 0.25 ml at each time point, 0.25 ml of fresh medium \pm colcemid was added to each plate of cells. At the end of 3 h, cells were washed twice with depleted medium followed by a wash with 50 mM *N*-acetyl-D-galactosamine. An aliquot of this was counted to determine the amount of ^{125}I -ASOR bound to the external surfaces of cells. After a final wash in depleted medium, cells were dissolved in 1.0 ml of 0.2 M NaOH to measure intracellular levels of labeled glycoprotein. Calculations for the level of degradation products appearing in the medium were adjusted for dilution due to replacement with fresh medium.

Protein concentrations were measured by the standard Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) described by the manufacturer. Two-dimensional gel electrophoresis was performed according to the method of O'Farrell (35). Autoradiographs were scanned with a Zenith Soft Laser Scanning Densitometer (LKB Instruments, Inc., Gaithersburg, MD).

RESULTS

To determine how sensitive the rate of tubulin synthesis is to changes in microtubule polymer levels, it was first necessary to obtain populations of cells with different, relatively homogenous extents of microtubule disassembly. Stable extents of microtubule depolymerization were achieved by treating cells with low doses of colcemid over the long term (3–6 h). Immunofluorescence staining with anti-tubulin antibody demonstrated that at each drug concentration, individual mouse 3T6 fibroblasts responded nearly identically resulting in a relatively homogeneous population of cells with microtubules of reduced length. Examples at six colcemid concentrations are shown in Fig. 1. There was no qualitative differ-

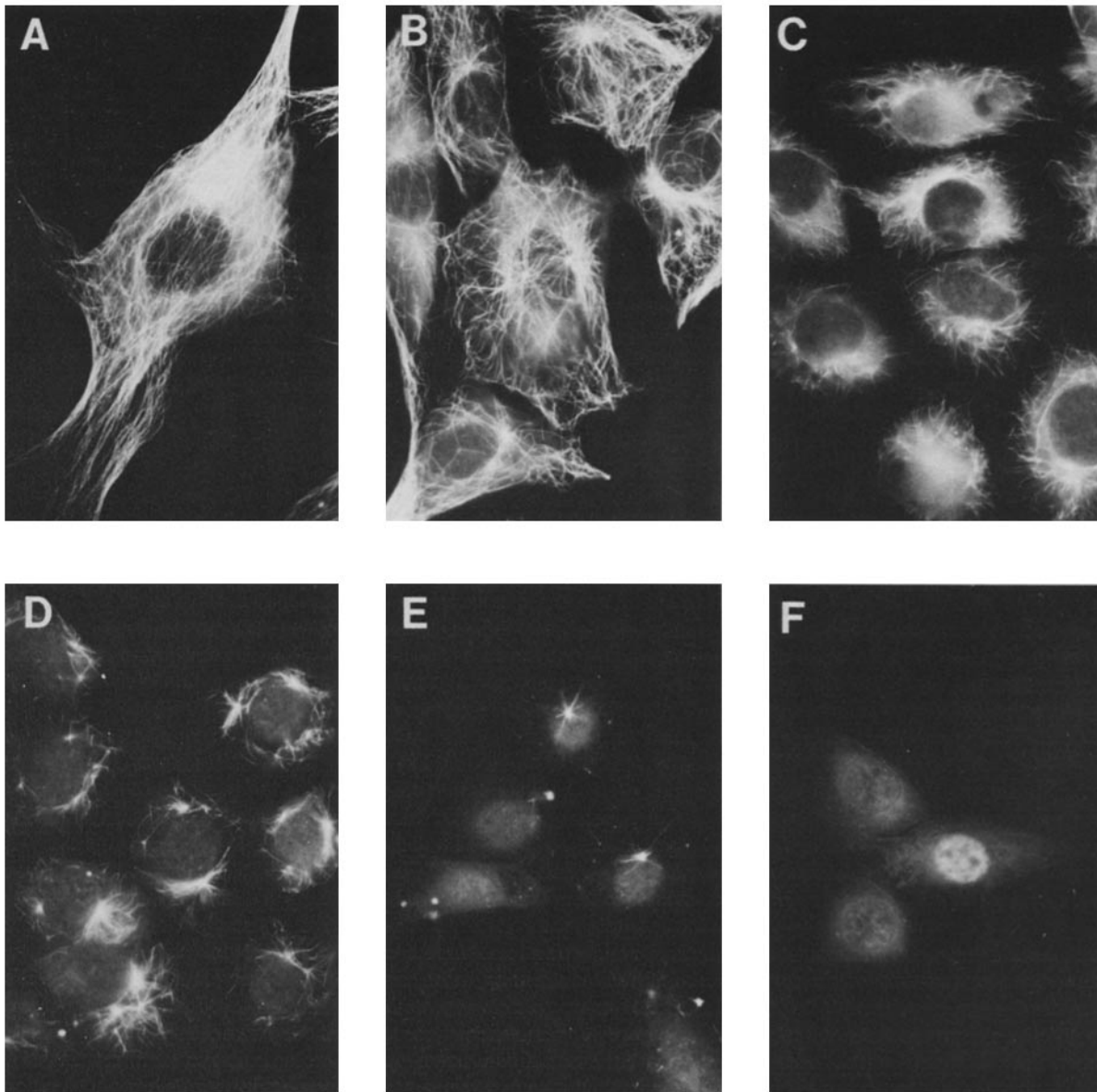


FIGURE 1 Immunofluorescence micrographs of microtubules in mouse 3T6 fibroblasts after controlled depolymerization. Cells were incubated with a range of colcemid concentrations for 3 h after which microtubules were visualized by indirect immunofluorescence with anti-tubulin antibody. (A) No colcemid. (B–F) 0.40, 0.80, 1.2, 2.4, and 12.0 μM colcemid, respectively.

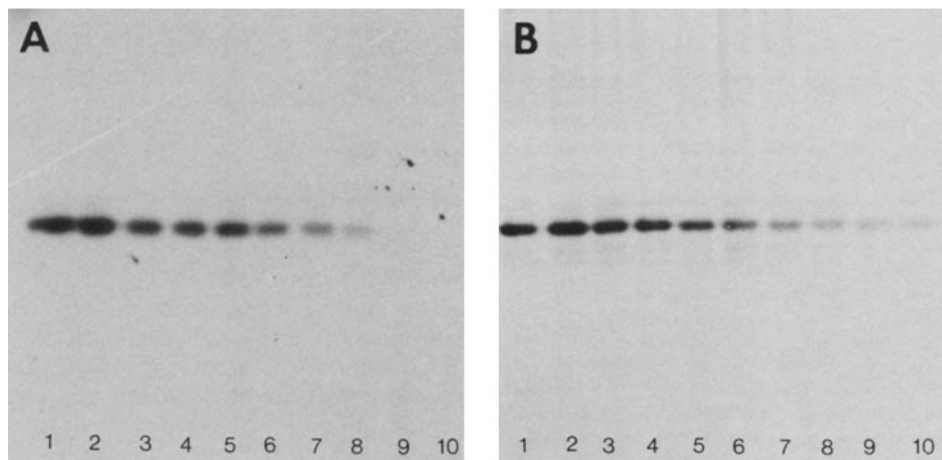


FIGURE 2 Quantitation of microtubule polymer levels and tubulin synthesis in mouse 3T6 fibroblasts after controlled depolymerization. (A) Autoradiograph of tubulin from cytoskeletons of cells treated with long term, low doses of colcemid. Fibroblasts were incubated with [³⁵S]methionine for 24 h. During the last 3 h of labeling, colcemid was added at various doses. Cells were then extracted with a microtubule-stabilizing buffer containing PEG-6000 to separate microtubule polymers from soluble tubulin. Immunoprecipitation was used to quantitate the amount of tubulin in the polymer fraction. (B) Autoradiograph of newly synthesized tubulin following treatment of cells with long term, low doses of colcemid. Fibroblasts were incubated with several concentrations of colcemid for 3 h. During the last 30 min, [³⁵S]methionine was added to label newly synthesized proteins. Cells were lysed and the relative rates of tubulin synthesis were quantitated by immunoprecipitation. (Lane 1) No colcemid; (lanes 2–10) 0.12, 0.24, 0.30, 0.40, 0.60, 0.8, 1.2, 2.4, and 12.0 μ M colcemid, respectively.

ence in the staining pattern between cells treated with colcemid for 3 h and cells treated for 6 h, further emphasizing that treatment with each drug concentration resulted in a relatively stable microtubule polymer mass (data not shown). Relative amounts of microtubule polymer remaining after incubation with colcemid were quantitated by immunoprecipitation as shown in Fig. 2A. For reasons which remain unclear, treatment of fibroblasts, as well as hepatocytes (see Fig. 4), with very low doses of colcemid consistently resulted in a small increase (~20%) in microtubule polymer levels. With the exception of this anomaly, each increasing colcemid concentration resulted in a progressive loss of microtubule polymer mass as expected from the immunofluorescence studies. Analysis of several experiments confirmed that this technique for quantitation of microtubule polymer levels could reproducibly discern small changes (10%) and thus allow the examination of the effect of subtle changes in polymer levels.

Sensitivity of Tubulin Synthesis to Small Changes in Microtubule Polymer Levels in Mouse Fibroblasts

It has been clearly demonstrated in several established mammalian cell lines and primary cultures that depolymerization of microtubules results in the turn-off of tubulin synthesis (3, 12). It remained unclear how sensitive the rate of tubulin synthesis is to changes in polymer levels. We, therefore, asked whether complete depolymerization of microtubules was required to decrease tubulin synthesis or if cells could alter the rate of synthesis in response to more subtle changes in polymer levels.

To examine the effects of limited microtubule depolymerization on tubulin synthesis, cells were incubated with various low doses of colcemid as in Fig. 1, subsequently incubated with [³⁵S]methionine for 30 min, and their rate of synthesis

was analyzed by immunoprecipitation. The autoradiograph of Fig. 2B demonstrates that with increasing colcemid concentrations, there was a progressive decrease in tubulin synthesis. When microtubule polymer mass and tubulin synthesis were compared at each drug concentration, it was apparent that these two parameters were, indeed, closely related (Fig. 3). From the results at low colcemid concentration, we concluded that the rate of tubulin synthesis is sensitive to even small changes in polymer levels.

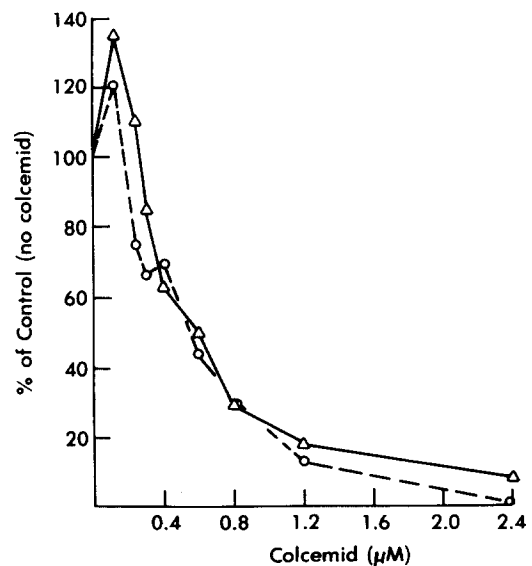


FIGURE 3 Comparison of microtubule polymer levels and tubulin synthesis in mouse 3T6 fibroblasts following controlled depolymerization. Band intensities of autoradiographs of Fig. 2, A and B, were measured by scanning densitometry. The level of polymer and synthesis in control cells was set at 100% and relative levels at each drug concentration were determined. O, Microtubule polymer; Δ , tubulin synthesis.

Response to Limited Microtubule Depolymerization in Rat Hepatocytes

We next sought to determine if the superimposition of microtubule polymer levels and tubulin synthesis found in mouse fibroblasts was a more general phenomenon. Primary cultures of rat hepatocytes were chosen because these cells represent a reasonably accurate model for a differentiated cell *in vivo*. For example, hepatocytes, when plated in monolayer, regain the structural polarity found *in vivo* and maintain most liver cell functions (25). This includes many functions which are thought to be microtubule dependent such as intracellular transport of hormones (25), immunoglobulins (17, 30), and lipoproteins (2, 46). To determine the optimal concentration range of colcemid to produce a gradation of microtubule polymer levels, hepatocytes were treated for 3 or 6 h with several concentrations of the drug, and microtubules were visualized by immunofluorescence (data not shown). Again, the response was fairly uniform from cell to cell. A range of colcemid was identified which produced microtubule polymer levels spanning from no detectable disassembly to complete depolymerization. This result was qualitatively similar to that found with fibroblasts although higher colcemid concentrations were required to alter microtubule lengths in hepatocytes. Also in contrast to fibroblasts, there appeared to be more disassembly of microtubules in hepatocytes treated with colcemid for 6 vs. 3 h at the higher drug concentrations ($>2.0 \mu\text{M}$). Once this range was established, immunoblot analysis was used to quantitate relative amounts of microtubule polymer remaining after incubation with colcemid. The resulting autoradiograph is shown in Fig. 4A. After autoradiography, the nitrocellulose blot was stained with Amido black to insure that equal amounts of protein from extracted cytoskeletons were analyzed (Fig. 4B). Relative rates of tubulin synthesis were quantitated by immunoprecipitation as described in Fig. 2B.

A comparison of the effects at each drug concentration is shown in Fig. 4C. As with fibroblasts, tubulin synthesis was found to be sensitive to small changes in microtubule polymer levels, indicating that this may be a general characteristic of autoregulation of tubulin synthesis.

Two quantitative differences between fibroblasts and hepatocytes were revealed when comparing the effects of altering microtubule polymer levels on the rate of tubulin synthesis. The first dealt with the exact shape of the dose-response curve. The second dealt with a much higher residual level of synthesis in hepatocytes at high colcemid concentrations. First, at low extents of depolymerization, tubulin synthesis is inhibited more strongly in hepatocytes than in fibroblasts. For example, when 10% of the microtubules had been disassembled, tubulin synthesis in hepatocytes was reduced by $\sim 55\%$ (Fig. 4C; $1.2 \mu\text{M}$ colcemid), whereas in fibroblasts, synthesis decreased only 10% (Fig. 3).

One explanation for the different responses of these two cell types was that hepatocytes and fibroblasts have different ratios of polymeric to soluble tubulin. If hepatocytes have a smaller percent of tubulin in the soluble form than do fibroblasts, then a small decrease in polymer would produce a larger percent shift in monomer level. (We note that the terms "soluble" and "monomeric" tubulin are defined here as all nonpolymerized tubulin protein). To test this possibility, the percent of soluble tubulin in both cell types was determined as described in Materials and Methods. We found that fibro-

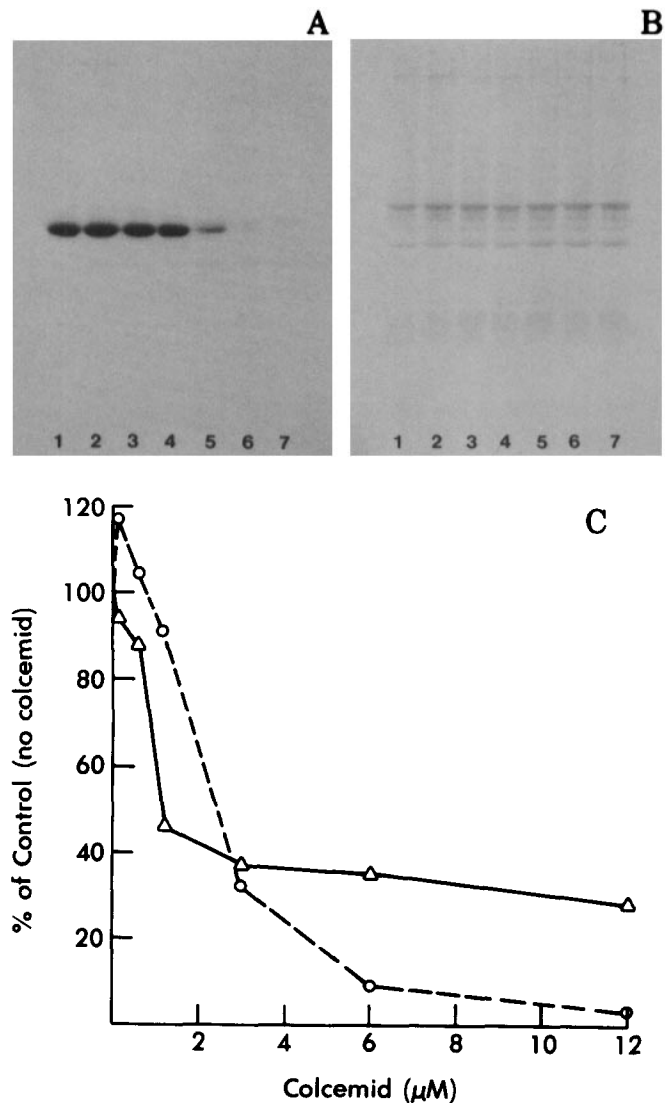


FIGURE 4 Comparison of microtubule polymer levels and tubulin synthesis in rat hepatocytes after treatment with various doses of colcemid. After incubation with colcemid for 3 h, hepatocytes were extracted in a microtubule-stabilizing buffer containing glycerol and microtubule polymer levels were quantitated by immunoblot analysis. Tubulin synthesis was measured by immunoprecipitation as described in Fig. 2B. (A) Autoradiograph of tubulin from cytoskeletons of cells treated with colcemid for 3 h. (Lane 1) No colcemid. (Lanes 2-7) 0.12, 0.6, 1.2, 3.0, 6.0, and $12.0 \mu\text{M}$ colcemid, respectively. (B) Nitrocellulose blot of Fig. 4A stained with Amido black after autoradiography. (C) Comparison of microtubule polymer levels and tubulin synthesis. Autoradiographs were scanned and levels of control were set at 100%. O, microtubule polymer; Δ, tubulin synthesis.

blasts contained 45 and 47% of total cellular tubulin in the polymer form (in two experiments using the immunoprecipitation method with PEG-6000 in the stabilization buffer), whereas in hepatocytes the percent of polymerized tubulin was found to be $\sim 85\%$ in six experiments using different experimental protocols (as shown in Table I). Theoretically, addition of low doses of colcemid to reduce total microtubule polymer levels by 10% would result in an increase of $\sim 60\%$ in the level of soluble tubulin in hepatocytes but only an increase of $\sim 20\%$ in fibroblasts. Examination of Figs. 3 and 4C ($1.2 \mu\text{M}$ colcemid) suggests that the rate of tubulin synthesis did, in fact, change to the same degree as the respective

TABLE I. Percent Polymer in Rat Hepatocytes Maintained in Primary Culture

	Method of analysis	
	Immunoprecipitation	Immunoblot
	%	%
Glycerol	90	84
PEG-6000	—	90
	80	87
	72	—

Hepatocytes were extracted with a microtubule-stabilizing buffer containing either 2 M glycerol or 4% PEG-6000. Percent polymer was then determined by either immunoprecipitation or immunoblot analysis as described in Materials and Methods. Each percent represents one experiment.

levels of soluble tubulin in the two cell types. By the same reasoning, the rate of tubulin synthesis cannot be correlated with the level of microtubule polymer. A 10% decrease in polymer levels would not result in striking differences between fibroblasts and hepatocytes. Although certainly not conclusive, these results support the hypothesis that inhibition of tubulin synthesis is modulated by the level of soluble tubulin.

A second difference between the two cell types is that complete depolymerization of microtubules in hepatocytes with colcemid did not reduce tubulin synthesis below ~30% of that measured in untreated cells, while tubulin synthesis in fibroblasts decreased to nearly undetectable levels. It is assumed that decreasing the polymer level with colcemid results in a stable increase in soluble tubulin. However, this is only true if rate of tubulin degradation is not accelerated. While treatment of fibroblasts with 12 μ M colcemid produces no significant change in tubulin protein half-life (~48 h; data not shown), depolymerization of microtubules in hepatocytes reduced the half-life of tubulin ~25-fold (from 50 to 2 h). This is shown in Fig. 5. Treatment of hepatocytes with nocodazole or colchicine produced similar results whereas taxol, which stabilizes microtubules, increased the half-life of tubulin (data not shown). The effect of colcemid seems to be specific for tubulin since the drug did not alter the turnover of proteins in general as determined by measuring trichloroacetic acid-precipitable counts. Furthermore, when radiolabeled proteins were analyzed by two-dimensional gel electrophoresis followed by fluorography, proteins from colcemid and control cells were virtually identical except, of course, for a decrease in the level of radiolabeled tubulin in drug-treated cells (data not shown).

As expected, the increased rate of tubulin degradation quickly reduced the level of total tubulin in hepatocytes (Fig. 6). After a 6-h incubation with colcemid, the level of tubulin was reduced by ~70%. It is apparent that the combination of a drastically shortened half-life of tubulin and the turn-off of tubulin synthesis induced by microtubule depolymerization caused the net loss of tubulin in hepatocytes. Hence the monomer level was continually being depleted, presumably leading to a constant albeit reduced rate of synthesis.

Sensitivity of Endocytotic Pathways to Small Changes in Microtubule Polymer Levels

As with studies on tubulin synthesis, most reports which suggest a role for microtubules in the control of specific cellular functions have been approached by first treating cells with high enough doses of anti-microtubule drugs to com-

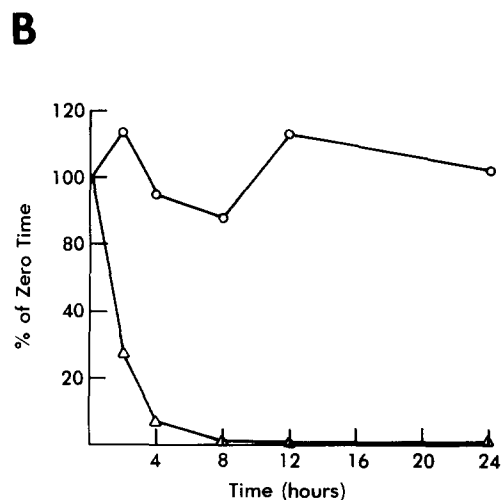
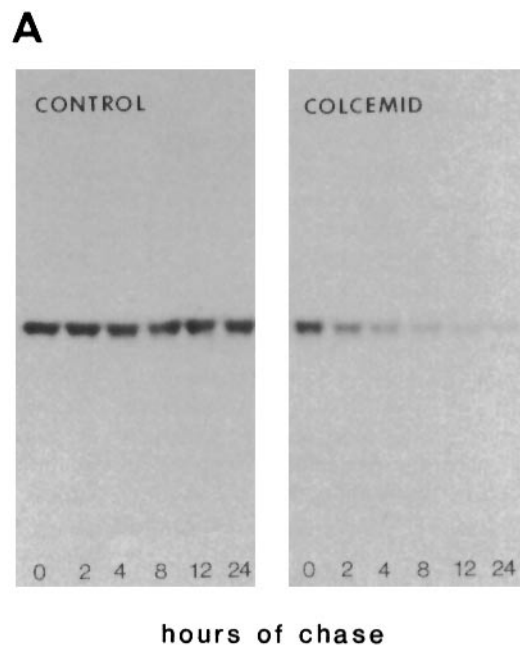


FIGURE 5 Effect of colcemid on the half-life of tubulin protein in rat hepatocytes. Cells were incubated for 22 h with labeling medium (depleted medium with 3 mg/liter methionine, 25 μ Ci/ml [35 S]-methionine), washed three times with L-15 salts at 37°C and replaced with depleted medium containing 30 mg/liter methionine \pm 12 μ M colcemid. Relative levels of radiolabeled tubulin remaining during the chase were determined by immunoprecipitation. Autoradiographs were scanned and levels of control cells at zero time (start of chase) were set at 100%. (A) Autoradiograph of immunoprecipitated tubulin protein at various times during the chase. (B) Percent of radiolabeled tubulin vs. time of chase. O, control cells; Δ , colcemid-treated cells.

pletely depolymerize the microtubules, and next determining which functions are no longer performed. In view of the results with tubulin biosynthesis, it was of interest to examine exactly how sensitive a presumed microtubule-dependent function was to more subtle changes in polymer levels. A role for microtubules in the processing of endocytosed ligands has been well documented (27, 29, 36). In particular, rat hepatocytes in monolayer culture have proved to be an excellent model for the study of endocytic pathways. Much of the work

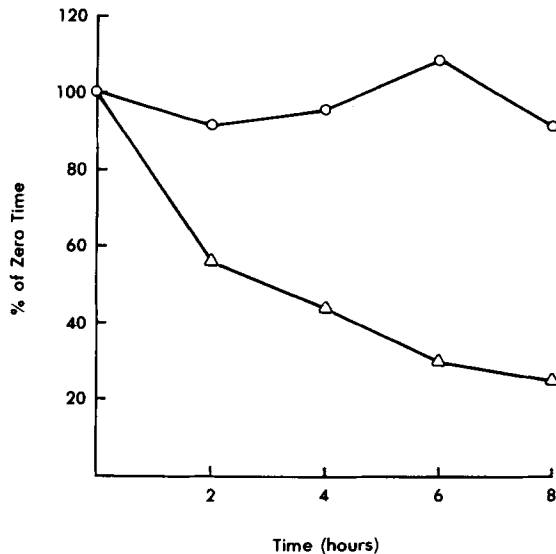


FIGURE 6 Effect of colcemid on the level of total cellular tubulin in rat hepatocytes. Cells were incubated in the presence and absence of $12 \mu\text{M}$ colcemid. At time points from 0 to 8 h, cells were lysed and the relative levels of total cellular tubulin were quantitated by immunoblot analysis. Autoradiographs were scanned and the level of control cells at zero time was set at 100%. O, control cells; Δ , colcemid-treated cells.

with cultured hepatocytes has focused on receptor-mediated endocytosis and processing of asialo-glycoproteins (20). These ligands are rapidly endocytosized by liver cells, separated from receptors, and degraded in lysosomes. The degradation products are finally secreted back out into the medium. Recently, Wolkoff et al. (50) detailed different steps in hepatic processing of the glycoprotein, ASOR, including a step which apparently requires microtubules, the intracellular segregation of receptor and ligand. With this background in mind we chose to examine the effect of small changes in microtubule polymer levels on the uptake and processing of ASOR in primary cultures of rat hepatocytes.

To measure endocytosis and degradation of the glycoprotein at various polymer levels, rat hepatocytes were preincubated with several doses of colcemid, pulsed 10 min with ^{125}I -ASOR at 37°C , washed in the presence of the drug, and the rate of degradation was measured. Control experiments demonstrated that colcemid had no effect on the binding or uptake of ^{125}I -ASOR into hepatocytes. Therefore, counts accumulating in the medium were due to degradation products of the endocytosed glycoprotein. As shown in Fig. 7, with each increase in colcemid concentration there was a concomitant decrease in the amount of degraded ^{125}I -ASOR suggesting that some step(s) in the processing of the endocytosed glycoprotein was sensitive to changes in microtubule polymer levels.

Using duplicate cell cultures, microtubule polymer mass was quantitated by immunoblot analysis after both a 3- and 6-h preincubation with the various colcemid concentrations (data for 3 h is shown in Figs. 4 and 8; data for 6 h is not shown). The 3-h incubation corresponded to the time immediately following the ^{125}I -ASOR pulse; i.e., the zero point of the degradation assay, while incubation with colcemid for 6 h was equivalent to the final time point of the assay. Comparison of the two incubation periods demonstrated that similar amounts of microtubule polymer were maintained in

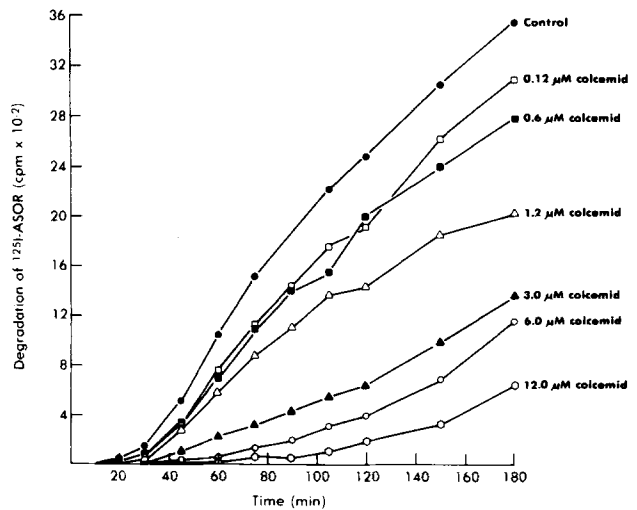


FIGURE 7 Effect of controlled depolymerization of microtubules on the processing of endocytosed ASOR in rat hepatocytes. Hepatocytes were treated with different doses of colcemid for 3 h. In the continued presence of the drug, ^{125}I -ASOR was added for 10 min at 37°C . Processing was assayed by measuring the level of degradation products in the medium.

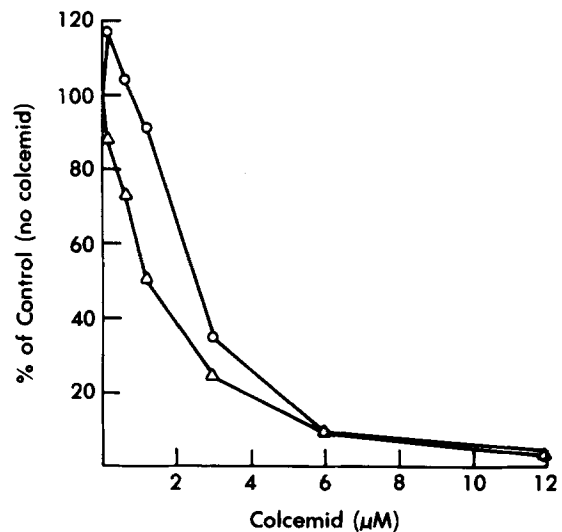


FIGURE 8 Comparison of microtubule polymer levels and processing of endocytosed ASOR in rat hepatocytes. After incubation with colcemid for 3 h, cells were extracted in a microtubule-stabilizing buffer containing glycerol and levels of polymer were measured by immunoblot analysis (data for polymer levels is also presented in Fig. 4). Microtubule polymer levels were compared to the level of degradation products appearing in the medium 75 min after incubation with ASOR (Fig. 7). Both parameters from control cells were set at 100% and relative levels in colcemid-treated cells were determined. O, microtubule polymer; Δ , processing of endocytosed ASOR.

cells after 3 and 6 h with low doses of colcemid ($<2.0 \mu\text{M}$) although there was somewhat less polymer in the 6-h samples at higher drug concentrations. Nevertheless, this comparison indicated that microtubule polymer levels in cells treated with low doses of colcemid were not rapidly changing during the course of the experiment. The relative amounts of degradation products appearing in the medium 75 min after the ^{125}I -ASOR pulse were compared to microtubule polymer mass at each colcemid concentration (Fig. 8). As demonstrated with

tubulin synthesis, treatment of hepatocytes with low doses of colcemid caused a marked inhibition of processing of endocytosed ligands, suggesting that some aspect of this function is dependent on microtubule polymer levels.

DISCUSSION

When fibroblasts or hepatocytes were treated with low doses of microtubule-depolymerizing drugs such as colcemid, there was a decrease in the level of microtubule polymer to a new, relatively stable state. Although individual cells varied somewhat in their extent of depolymerization, the overall population behaved in a remarkably uniform manner as visualized by anti-tubulin immunofluorescence. This uniform behavior has allowed us to examine the response of cells to controlled depolymerization of their microtubules.

To study microtubule polymer levels in populations of cells, we have used a modification of techniques developed by Duerr et al. (14) whereby the polymer fraction can be separated from the monomer by extracting cells under conditions in which the polymer is stabilized. The level of polymer after controlled depolymerization was determined either by immunoprecipitation after cells were labeled over the long term with [³⁵S]methionine or by immunoblotting. Several control experiments indicate that polymer levels determined by these methods do, in fact, reflect levels found *in vivo*. First, using the immunoprecipitation method the percent of tubulin in the polymer fraction of mouse 3T6 fibroblasts was found to be 45 and 47% of total cellular tubulin in two different experiments. This is in close agreement with 40% polymer in mouse 3T3 fibroblasts reported by Hiller and Weber (23) whose techniques involved direct calculation of polymer levels from immunofluorescent pictures and thus did not depend on extraction of cells with a microtubule-stabilizing buffer. Second, we estimated that rat hepatocytes in primary culture contained ~85% of total cellular tubulin in the polymerized form. Demonstrating such a high percent of polymerized tubulin in hepatocytes indicated that there was no drastic disassembly of microtubules during the extraction procedure. Third, extraction of hepatocytes with microtubule-stabilizing buffers containing either PEG-6000, which has been shown to stabilize microtubules *in vitro* against serial dilution (5), or glycerol produced similar results (Table I).

In addition, immunofluorescence studies were used to analyze qualitatively the ability of stabilizing buffers to preserve microtubule arrays in fibroblasts and hepatocytes. Cells were treated with various doses of colcemid to produce a gradation of microtubule polymer levels and either fixed and stained with anti-tubulin antibody or first extracted in microtubule-stabilizing buffers containing either PEG-6000 or glycerol and then processed for immunofluorescence (data not shown). In either case, there were no qualitative differences between cells which had been immediately fixed or first carried through the extraction procedure. This demonstrated that microtubules of any length and number were, in fact, preserved during extraction.

The estimation of polymer mass was found to be highly reproducible. Although some variability is expected when using primary cultures, we measured polymer levels in hepatocytes to be ~85% in six experiments (Table I). This value for polymer is higher than the 15% previously reported for rat hepatocytes maintained in suspension as determined by morphometric electron microscopy (40). This difference is

most likely due to different conditions in which the cells were grown although morphometry may have underestimated polymer mass. It is known, for example, that during the first 24 h of culture, hepatocytes undergo morphological changes (6) as well as changes in transcription rates (26) and enzyme activities (33, 49). Furthermore, it is quite clear from several studies that hepatocyte substrate interactions and cell-cell contact will influence hepatocyte function (8, 18, 32, 42). In our experiments, we have cultured hepatocytes on a collagen substrate at high density and used the cells 48 h after preparation which may have better preserved microtubule polymer mass.

Tubulin Synthesis and Microtubule Polymer Levels

Most previous studies of microtubule function in cells have examined the response of a specific cell function after total depolymerization of microtubules with anti-microtubule drugs. However, except during mitosis when many functions cease (22, 43), cytoplasmic microtubules in most cell types do not fluctuate in such a dramatic manner, but presumably undergo more subtle changes. We, therefore, examined the question of whether tubulin feedback regulation is likely to be physiologically important during these processes. We have shown that the rate of tubulin synthesis in both mouse 3T6 fibroblasts and primary cultures of rat hepatocytes is sensitive to small changes in microtubule polymer levels. As polymer levels decreased, there was at least an equal decrease (3T6 cells) or a greater than equal decrease (hepatocytes) in the rate of tubulin synthesis. The possibility remained that inhibition of tubulin synthesis was not due to changes in the polymer/monomer ratio but was due instead to a direct effect of the antimicrotubule drug. Using a nonpharmacological approach, Cleveland et al. (13) showed that tubulin synthesis was suppressed when Chinese hamster ovary cells were microinjected with purified tubulin protein, thus demonstrating that tubulin is the effective species. However, microinjection itself increased the intracellular concentration of tubulin by ~50% which again is not a subtle manipulation of the tubulin pool. Our studies demonstrate that cells can respond to much smaller manipulations of the monomer level and therefore possess a sensitive mechanism for regulating the synthesis of tubulin. This mechanism is likely to operate in the physiological range of perturbations in the polymer mass and, therefore, is not merely a response to stress.

It has been suggested by previous experiments that it is the level of soluble tubulin which modulates the rate of tubulin synthesis (3, 12). Comparison of the response of fibroblasts to that of hepatocytes produced new evidence to support this hypothesis. Hepatocytes were shown to have only 15% of their total cellular tubulin in the soluble form in contrast to 55% soluble tubulin in fibroblasts. If the level of soluble tubulin does regulate the rate of tubulin synthesis, it is expected that the response of hepatocytes to low levels of microtubule depolymerization would be more extensive than the same degree of depolymerization in fibroblasts. For example, only a 10% decrease in polymer mass would increase the monomer content of hepatocytes by ~60% but would increase by only 20% the monomer content of fibroblast. As shown in Figs. 3 and 4, this was the case. A small decrease in microtubule polymer mass produced a much larger effect on tubulin synthesis in hepatocytes when compared to fibroblasts.

Another interesting difference between these two cell types is that the hepatocytes, but apparently not fibroblasts, responded to elevated monomer levels by degrading the non-polymerized tubulin protein. In hepatocytes, the half-life of tubulin polypeptides was reduced ~25-fold in response to complete microtubule depolymerization though the half-lives of total cellular proteins were not affected. The effect of low levels of microtubule depolymerization on the half-life of tubulin protein has not yet been determined. The physiological relevance of this result is unclear. It is possible that hepatocytes may have merely destroyed colcemid-tagged molecules. Alternatively, hepatocytes may tightly regulate the level of soluble tubulin. In support of the latter possibility, preliminary work indicates that total cellular tubulin levels, but not rates of tubulin synthesis, were decreased by long-term incubation of hepatocytes with insulin (Caron, J. M., unpublished observation) demonstrating that a physiological agent as well as colcemid can affect the half-life of tubulin protein. One explanation for the difference between fibroblasts and hepatocytes is that hepatocytes in culture maintain many liver cell activities including several which are microtubule dependent. Therefore, homeostatic controls in hepatocytes may be much more elaborate and require the maintenance of specific levels of polymer or soluble tubulin or both.

One major proposed function of microtubules in cells and in liver cells in particular concerns intracellular transport. It is known that processing (degradation) of endocytosed asialoglycoproteins in hepatocytes maintained in monolayer culture is sensitive to doses of colcemid which cause complete depolymerization of the interphase microtubule arrays (50). Using isolated rat hepatocytes in suspension, Oka and Weigel (36) have further shown that degradation, but not binding or endocytosis, of asialo-glycoproteins is sensitive to low concentrations of microtubule-depolymerizing drugs. We have quantitated both microtubule polymer mass and the rate and extent of degradation of the glycoprotein, ASOR, after treatment of primary cultures of rat hepatocytes with low doses of colcemid. As shown in Fig. 8, the degree of inhibition of degradation parallels the extent of microtubule assembly, suggesting that some microtubule-dependent process is rate limiting in the endocytotic pathway and further suggesting that the polymer/monomer ratio could regulate the rate of degradation. It is clear, however, that though rate limiting, microtubules are not essential for processing. Even at high enough doses of colcemid to completely disrupt microtubules, degradation products eventually accumulated in the extracellular medium. We suggest the following explanation. Using time-lapse video analysis, Herman and Albertini (21) have shown that endosomes in cultured rat granulosa cells initially underwent saltatory movements followed by gradual centripetal migration to the cell center where lysosomes had accumulated. We suggest that in hepatocytes, newly endocytosed vesicles containing ASOR likewise underwent saltatory movement until becoming entrapped in the microtubule network which then directed movement of granules to the cell center for degradation. Immunofluorescence staining of hepatocytes treated with various doses of colcemid showed that microtubule polymers were disassembled from the cell periphery towards the cell center (as shown in Fig. 1 for fibroblasts). Therefore, as microtubule polymer levels decreased, it would take longer for endosomes to reach the microtubule network, resulting in a decreased rate of appearance of degradation products. On

the other hand, complete depolymerization of microtubules in granulosa cells was shown to alter the distribution of lysosomes from a perinuclear position to a random arrangement in the cytoplasm (21). Therefore, we further suggest that at high doses of colcemid, lysosomes would eventually reach the cell periphery where interception and processing of endocytosed glycoprotein could take place. In this view, microtubules would regulate the accessibility of endocytic vesicles to lysosomes possibly by segregating them or providing an efficient method of transport.

In conclusion, we have shown that two microtubule-dependent functions, autoregulation of tubulin synthesis and processing of endocytosed ligands, are sensitive to small changes in microtubule polymer levels. These results strongly suggest (a) that autoregulation of tubulin synthesis operates within the physiological range of polymer/monomer ratios of interphase cells, and (b) that subtle changes in polymer levels are used as a fine control mechanism to regulate different cellular functions.

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