Synthesis and Degradation of Microtubule Protein in Synchronized Chinese Hamster Cells*

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SUMMARY

Synthesis, degradation, and colchicine-binding characteristics of the microtubule protein were assayed in the cell cycle of two Chinese hamster lines. Levels of microtubule protein in the cell cycle were determined by electrophoresis of purified iodinated microtubule protein together with tritium-labeled whole cell lysates. Synthesis occurred almost exclusively in the G2 phase of the cloned aneuploid V79 line, whereas in the diploid Don line, considerable microtubule protein was synthesized in mid S phase as well as G2. In both Don and V79 cells, colchicine-binding activity displayed a pattern which was in agreement with the changing levels of microtubule protein determined with polyacrylamide gels. Degradation of the protein appeared to be intermittent. Microtubule protein synthesized in G2 phase survived into the ensuing G1 phase but was catabolized rapidly and concomitantly with initiation of DNA replication. The results show an intracyclic rhythm in microtubule synthesis and colchicine-binding activity and give support to the idea that oscillatory behavior of proteins is a common characteristic of the mammalian cell cycle.

The microtubule protein is among the most ubiquitous of proteins and, being the major component of the mitotic apparatus, is of obvious importance in rapidly dividing cells. In addition to its role in cell division, it has been shown to function in motility, secretion, transport, and in the maintenance of morphology (1-10). In the developing chicken brain it can represent 40% of the soluble and 25% of the total protein (11), and in cultured cells it represents 15% of the soluble protein (9). These characteristics of the microtubule protein make it an especially desirable protein for the study of regulation in the mammalian cell cycle.

Earlier studies with synchronized mammalian cell lines showed that a number of enzymes necessary for intermediary metabolism (13-15) and for nucleic acid synthesis (16-18) exhibited periodic behavior during the cell division cycle. The pattern of fluctuations in enzyme levels was different in aneuploid and diploid cells and appeared to be temporally coordinated with changes in the rate of DNA synthesis (16). The fact that periodic maxima were displayed by a large number of enzymes and were cell cycle dependent suggested that degradation as well as synthesis might be an important component of the regulatory system.

MATERIALS AND METHODS

Culture—A diploid Chinese hamster cell line, Don, and a cloned aneuploid Chinese hamster line, V79, were used in this study. Don was derived from a male hamster lung (19) and V79 was derived from a female hamster lung (20). The procedures for growing cells and for colcemid synchronization have been described (16). Cells were also synchronized with a simple mitotic selection without colcemid (21).

Radioactive Labeling—Proteins were labeled with [3H]RPH (1 μCi per ml; mean specific activity, 700 mCi per mmole; Schwarz BioResearch). The rate of DNA synthesis was asayed by pulsing the cells for 15 min with [methyl-3H]thymidine (5 μCi per ml, 5 Ci per mmole, Amersham-Searle), or by pulsing for 30 min with [3H]thymidine (0.05 μCi per ml, 55.7 mCi per mmole, ICN Tracerlab). Cells were grown in scintillation vials (3 × 10⁶ cells per vial), and a procedure for DNA extraction similar to that of Klevecz and Stubblefield was used (22). At hourly intervals the medium was drawn off and replaced with labeled medium for the time period described above. At the end of the pulse, the cells were washed three times with 5 ml of Hanks' balanced salt solution, extracted three times with 5 ml of 10% trichloroacetic acid, and washed two times with 5 ml of ethanol-ether (1:1) and once with 10 ml of ether. The samples were air-dried, scintillation fluid was added, and they were counted in a Beckman LS230 liquid scintillation counter. All time points were assayed in triplicate. Microtubule protein was labeled with ¹²⁵I according to the procedure of McConahey and Dixon (23). In this work, the term previous labeling refers to experiments in which isotope was added to randomly growing exponential cultures prior to synchronization.

Microtubule Protein Purification—Microtubule protein was purified from mouse and Chinese hamster brain using method number two described by Weisenberg et al. (24). Polyacrylamide Gels—Samples were reduced with 0.01 M dithiothreitol or 0.1 M mercaptoethanol prior to electrophoresis.

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The abbreviations used are: RPH, reconstituted protein hydrolysate; [3H]RPH, tritium-labeled RPH; SDS, sodium dodecyl sulfate.
Gel electrophoresis was performed in 10% acrylamide gels with 0.1% SDS according to the procedure of Laemmli (25). Microtubule protein was also analyzed in 7.5% acrylamide-8 M urea gels at pH 8.4 with the technique of Russell et al. (26). The reservoir buffer in both systems was Tris-glycine (1.5 g per liter of Tris, 7.2 g per liter of glycine, pH 8.4). The urea gels were stained for 3 hours with 1% Amido black and destained overnight in a diffusion destainer.

**Microtubule Analysis on Polyacrylamide Gels**—Cells were disrupted by sonication, centrifuged for 1 hour at 114,000 X g, and a constant amount of 3H-labeled microtubule protein (approximately 100 to 150 cpm) was added as a marker to each supernatant sample. The samples were reduced with 0.1 M mercaptoethanol or 0.01 M dithiothreitol in a boiling water bath for 2 to 3 min prior to electrophoresis. One sample containing only 3H-labeled microtubule protein was treated identically with the other samples. After electrophoresis, the gels were frozen and fractionated with a razor blade (1-mm slices) or with a Micell gel slicer (Brinkmann Instruments, Inc.) which gave uniform 0.5-mm slices. Samples were counted for 3H in a Nuclear Chicago γ counter to locate the peak of the microtubule band. The peak slices were then hydrolyzed with 30% hydrogen peroxide overnight at 65–70°C or at 50–55°C according to the procedure of Bakay (27). The samples were dissolved either in 0.5 ml of NCS solubilizer (Amersham-Searle) with the addition of 10 ml of scintillation fluid or in 10 ml of Aquasol scintillation fluid. The samples were counted to 2% standard error. The percentage of 3H counts in the tritium channel was determined from the 3H-labeled microtubule sample. This value was subtracted from the tritium-labeled samples to obtain the net tritium counts in the microtubule protein.

**Colchicine Binding**—Synchronized cells were collected at hourly intervals and resuspended into 0.5 ml of buffer (0.01 M sodium phosphate, 0.1 M sodium glutamate, pH 6.8). Lysates were prepared by sonication and centrifuged for 30 min at 15,000 X g. A colchicine-binding decay curve was determined on the supernatant as described by Wilson (28). The supernatant solutions were divided into three fractions of 0.15 ml each. These were incubated at 37°C for 1, 2, and 3 hours. 3H]Colchicine (2.7 X 10^4 M final concentration) was added to the samples during the last hour of each incubation period. After 1 hour the first fraction was transferred to 0°C and unlabeled colchicine was added (5.7 X 10^4 M final concentration) to stop additional binding of labeled colchicine and to lower the background of the filter-binding assay. The process was repeated for the 2- and 3-hour sample. Each 0.15-ml fraction was divided into three 0.05-ml aliquots and spotted on moistened discs of Whatman DE81 paper. After 10 min, the discs were washed five times with buffer (0.01 M sodium phosphate, 0.1 M sodium chloride, pH 6.8). The discs were counted in 5 ml of Aquasol (New England Nuclear). Colchicine-binding activity was determined by assuming first order decay kinetics to extrapolate the decay curve to zero time (28).

**RESULTS**

**Synchronization**—Synchrony was monitored by measuring the DNA synthetic rate and by scoring mitotic indices. The mitotic indices ranged from 95 to 98% at the beginning of each experiment. Fig. 1 shows the DNA synthetic rate for a number of experiments with V79 and Don cells. The rates have been plotted so that the peaks of each curve coincide. The anaploid V79 line shows a unimodal DNA synthetic pattern and the diploid Don line shows a characteristic biphasic pattern. These differences have been described previously for heteroploid and diploid cell lines (16).

**Identification of Microtubule Protein**—Microtubule protein from Chinese hamsters was isolated on polyacrylamide-SDS gels by using purified mouse 125I-labeled microtubule protein as a marker. This marker protein and purified unlabeled Chinese hamster microtubule protein migrated identically in polyacrylamide-SDS gels (Fig. 2b). The 125I-labeled microtubule marker was then used to identify the microtubule protein band in a 114,000 X g supernatant extract from Don cells (Fig. 2a). Other experiments with V79 and Don cells have shown that the microtubule protein band in Don cells is a major component of the 114,000 X g supernatant fraction. Similar results have also been shown for brain extracts in which microtubule protein accounts for 10 to 40% of the soluble protein (11, 29).

**Microtubule Assay**—V79 cells were labeled for 24 hours with [3H]RPH (1 μCi per ml). The cells were sonicated and centrifuged to obtain the 114,000 X g supernatant fraction. Increasing amounts of the supernatant fraction were analyzed on polyacrylamide-SDS gels with a constant amount of 3H-labeled microtubule marker. Fig. 3 shows that a linear relationship exists between the radioactivity and the amount of microtubule protein in the supernatant fraction. The amount of supernatant extract analyzed in Fig. 3 covers the range of the amount used in the synchrony experiments. This procedure allows the measurement of labeled microtubule protein with a maximum variation of 20%.

**Microtubule Synthesis in Synchronized V79 Cells**—Exponential V79 cell cultures were labeled for 21 generations prior to synchronization with medium containing [3H]RPH (1 μCi per ml). After the cells were synchronized, they were again resuspended in medium containing [3H]RPH at 1 μCi per ml. This procedure insured that the protein, the intracellular pools, and the medium were labeled to constant specific activity (30). Consequently, the amount of label incorporated directly reflected the level of microtubule protein. At hourly intervals following synchronization, cells were disrupted by sonication and aliquots of the tritium-labeled, 114,000 X g supernatants were analyzed on SDS-polyacrylamide gels along with a constant amount of the 3H-labeled microtubule marker. The counts from the trit
Fig. 2. Analysis of microtubule protein on polyacrylamide-SDS gels. a, gel scan of a 114,000 × g supernatant fraction of Don cells (---) analyzed on 10% polyacrylamide-SDS gels with purified mouse 125I-labeled microtubule protein (---). The high background (30% of peak height) of the gel scan is due to the staining procedure and does not represent the amount of background obtained with the labeled cell extracts (10 to 15% of peak height). Electrophoresis was performed at 150 volts for 5 hours. b, gel scan of purified Chinese hamster microtubule protein analyzed on 10% polyacrylamide-SDS gels (---) with mouse 125I-labeled microtubule protein (---). Electrophoresis was performed at 250 volts for 3½ hours.

Fig. 3. Analysis of microtubule protein from a 114,000 × g extract on polyacrylamide-SDS gels. V79 cells were labeled with 125I]RPH (1 pCi per ml) for 24 hours. Increasing amounts of 114,000 × g extract were analyzed on polyacrylamide-SDS gels with 125I-labeled microtubule protein. The solid line was calculated with the method of least squares. Dashed lines indicate one standard deviation from the least squares fit.

Microtubule protein from V79 cells and Don cells was analyzed on 10% polyacrylamide-SDS gels as described under "Materials and Methods." Mitotic cells were collected with and without prior colcemid block as described under "Materials and Methods." Analyzed were 6 × 10⁶ cells per sample in the colcemid block experiment (---) and 3 × 10⁶ cells per sample in the mitotic selection experiment (O---O).
is due to an increased rate of microtubule catabolism. It is certainly suggestive from the fluctuations observed in microtubule protein level in diploid Don cells that considerable protein catabolism is occurring.

In Don cells which were previously labeled for 24 hours and subcultured into labeled medium, 40% of the radioactivity in microtubule protein (O-O) on polyacrylamide-SDS gels. Gels were stained with 1% Amido black and the microtubule band was extracted in NCS hot acid-soluble fractions (X-X) analyzed as described previously (30).

The microtubule band was identified in Amido black-stained gels, and as might be expected from the results shown in Fig. 5c, the absorbance values of this band decreased as the cells entered S phase. The resolution of this technique, however, was not sufficient to permit extensive interpretation.

**Colchicine Binding**—Apparent levels of microtubule protein determined by analysis of polyacrylamide gels may be elevated above actual levels due to nonmicrotubular protein: which migrates close to or at the same position in the gels. However, nonmicrotubular protein contamination must be minimal because microtubule protein accounts for 15 to 40% of the soluble protein. Moreover, analysis was performed on 0.5-um fractions from the center of the 3H peak, thus minimizing cross-contamination. Additional support for the polyacrylamide gel studies comes from the following experiments in which colchicine binding was used to analyze for microtubule protein.

Since colchicine specifically binds to microtubule protein, this binding has been used to assay for the presence of microtubule protein (1, 28). Fig. 6 shows the linear increase that is obtained with the [3H]colchicine-binding assay. Wilson has shown that estimates of microtubule level can be obtained from the decay kinetics of this protein at 37°C. If multiple point binding assays are performed, the initial colchicine-binding activity can be determined by extrapolating the decay curve to zero time (28). The data presented in Fig. 7 show the microtubule decay
Fig. 6. Colchicine binding in the 114,000 × g supernatant extract. Colchicine binding was determined on increasing amounts of 114,000 × g V79 supernatant extracts. A linear relationship exists between colchicine binding and amount of microtubule protein. Maximum variations of 15% were observed.

FIG. 7. Decay of colchicine-binding activity at 37°. Incubated at 37° were 15,000 × g extracts from synchronized V79 cells, and the colchicine-binding activity was determined as described under "Materials and Methods." First order decay kinetics was assumed (28, 33), and a least squares fit was calculated. The colchicine binding at zero time was calculated for each hour and is shown in Fig. 8a for V79. Fig. 8b shows the calculated results for the diploid Don line with the same procedure. The Don cells showed major peaks of colchicine binding in mid S and G2 while the V79 cells showed the major binding peak in G2. Considerable binding activity has been found in the particulate fraction of neuroblastoma cells (9) and consequently it is possible that oscillations in binding activity could result from aggregation of microtubule protein from the supernatant solution into the particulate fraction. This possibility was minimized by analyzing the total cell lysate from Don cells (Fig. 5) on polyacrylamide gels and comparing the results to the colchicine binding on the 114,000 × g supernatant solution (Fig. 8b). The results were the same in either case. In addition, single point colchicine binding on the total cell lysate also showed a periodicity in activity in Don cells during mid S in agreement with the polyacrylamide gel results. Finally, in the two cell lines used in this study colchicine-binding activity of the particulate fraction was only 5% of the binding activity of the soluble fraction. Thus, the colchicine binding data agree with the results from polyacrylamide gels.

DISCUSSION

A diploid cell line and an aneuploid cell line from Chinese hamsters were used in a study of microtubule protein synthesis through the cell cycle. Synthesis of microtubule protein occurred almost exclusively in the G2 phase of the cell cycle in the cloned aneuploid V79 cells, while in the diploid Don cells a considerable amount of microtubule protein was synthesized in mid S as well as in G2. Klevecz (16) has described differences in DNA and enzyme patterns in the cycle of diploid and non-diploid cells and has suggested that many enzymes in heteroploid cells would show a simpler activity pattern than would...
homogeneous diploid cells, and in particular, that peaks in activity would be seen only in G1 and G2, if at all. Recently, Churchill and Studzinski (18) working with synchronous HeLa cells found this to be the case for DNase and alkaline phosphatase. However, in several other synchrony studies enzyme activity and rates of RNA and protein synthesis have been reported to be nearly linear functions of time in the cycle (34, 35). The results presented here for the cloned V79 aneuploid cell suggest that periodic synthesis is a general characteristic of the cell cycle and that failure to detect such a pattern may be due to population heterogeneity.

Robbins and Shelanski (36) reported that the level of colchicine-binding protein per cell was essentially the same at three points in the HeLa cell cycle, whereas we found considerable variation in binding activity through the Chinese hamster cell cycle. The degradation of functional proteins, while seemingly uncorporate p-fluorophenylalanine. They suggested that the duration of mitosis could be increased by allowing cells to incorporate p-fluorophenylalanine. They suggested that the conservation of the p-fluorophenylalanine in a mitosis-associated protein. Our results have shown that most of the microtubule protein was synthesized in mid S and G2 and are, therefore, in agreement with those of Sisken and Wilkes. However, direct measurements of microtubule protein half-life did not support the idea that this protein is conserved, and it is possible that these workers may have been measuring an effect on a stable protein distinct from microtubule protein.

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