Mutations in α- and β-Tubulin Affect Spindle Formation in Chinese Hamster Ovary Cells

IRENE ABRAHAM, MENASHE MARCUS,* FERNANDO CABRAL,* and MICHAEL M. GOTTESMAN
Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda,
Maryland 20205; *Department of Genetics, Hebrew University, Jerusalem, Israel; and *Division of
Endocrinology, University of Texas Medical School at Houston, Houston, Texas 77025

ABSTRACT Two Chinese hamster ovary cell lines with mutated β-tubulins (Grs-2 and Cmd-4) and one that has a mutation in α-tubulin (Tax-1) are temperature sensitive for growth at 40.5°C. To determine the functional defect in these mutant cells at the nonpermissive temperature, they were characterized with respect to cell cycle parameters and microtubule organization and function after relatively short periods at 40.5°C. At the nonpermissive temperature all the mutants had normal appearing cytoplasmic microtubules. Premature chromosome condensation analysis failed to show any discrete step in the interphase cell cycle in which these mutants are arrested. These cells, however, show several defects at the nonpermissive temperature that appear related to the function of microtubules during mitosis. Time-lapse studies showed that mitosis was lengthened in the three mutant lines at 40.5°C as compared with the wild-type cells at this temperature, resulting in a higher proportion of cells in mitosis after temperature shift. There was also a large increase in multinucleated cells in mutant populations after incubation at the nonpermissive temperature. Immunofluorescent studies using a monoclonal anti-α-tubulin antibody showed that the mutant cells had a high proportion of abnormal spindles at the nonpermissive temperature. The two altered β-tubulins and the altered α-tubulin all were found to cause a similar phenotype at the high temperature that results in mitotic delay, defective cytokinesis, multinucleation, and ultimately, cell death. We conclude that spindle formation is the limiting microtubule function in these mutant cell lines at the nonpermissive temperature and that these cell lines will be of value for the study of the precise role of tubulin in mammalian spindle formation.

Mitosis was one of the first cellular phenomena studied at the level of the light and the electron microscope, but relatively little is known of its molecular biology. The spindle, a morphologically prominent structure during mitosis, is composed of microtubules that are principally polymerized dimers of α- and β-tubulin (9). The biochemistry of microtubules in vitro has been well studied, but much less is known about the way in which microtubules form spindles in vivo during mitosis. One approach to this problem that has become feasible in the last few years is the study of mammalian cell lines carrying mutations that specifically affect α- and β-tubulin (4, 6, 16, 20). Another approach has been to study temperature-sensitive cells blocked in mitosis or cytokinesis (e.g., 11, 26, 28, 30, 32, 33).

Three mutant Chinese hamster ovary (CHO) cell lines that have alterations in β-tubulin (6), and one mutant with an alteration in α-tubulin (4) have been isolated in our laboratory. One β-tubulin mutant, Cmd-4 (10193), was isolated on the basis of resistance to the microtubule-disrupting drug colcemid; a second β-tubulin mutant, Grs-2 (10132), which carries a different alteration in β-tubulin, was selected as a griseofulvin-resistant clone. The α-tubulin mutant, Tax-1 (10576), was selected for resistance to the microtubule stabilizing drug taxol. All of the mutant cells are heterozygotes in the sense that the mutant genes are expressed together with

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1 Abbreviations used in this paper: CHO, Chinese hamster ovary; PCC, prematurely condensed chromosomes.
at least one normal functional α- or β-tubulin gene (4, 6).

Each of these tubulin mutants has been demonstrated to be temperature sensitive for growth at 40.5°C (4, 5). Temperature sensitivity for a β-tubulin mutant (ben A33) has also been reported in *Aspergillus nidulans* (22). This mutation causes a block in nuclear division and nuclear movement at the nonpermissive temperature. In this work, we present morphological and cell cycle studies to explore the basis of the cell death of our CHO tubulin mutants at the nonpermissive temperature. We have previously shown that in the case of mutant Cmd-4, the presence of the mutated β-tubulin does not grossly affect the structure or distribution of cellular microtubules (5). The present study demonstrates that these α- and β-tubulin mutations do have a deleterious effect on the mitotic spindle, resulting in increased mitotic index, failure of cytokinesis, and multinucleation at the nonpermissive temperature.

**MATERIALS AND METHODS**

**Cell Lines and Culture:** The wild-type parental cell line (10001 or 10004) is a subclone of the CHO cell line Pro 5 (29). The β-tubulin mutants Cmd-4 (10193) and Grs-2 (10132) and the α-tubulin mutant Tax-1 (10576) were derived from the wild-type strain after ethylmethane sulfonate mutagenesis (10193, 10132) or UV-irradiation (10576) and were selected on the basis of cell death of our CHO tubulin mutants at the nonpermissive temperature. Mitotic cells were identified by the presence of condensed chromosomes. Approximately 100 cells were stained by autoradiography with 2% aceto-orcein or after autoradiography with 2% Giemsa's.

**Mitotic Index:** Cells were plated at 5 x 10^4 per 100-mm tissue culture plate and were cultured at 37° or 40.5°C for various periods. After appropriate incubation, cells were collected, swollen in 0.075 M KCl, fixed, spread, and stained with Giemsa's as for standard karyotyping. Approximately 10^3 cells were spread per slide and mitotic index was determined by examining the number of mitotic cells among the first 1,000 scored cells. Cells were counted without knowledge of cell type or temperature regime to avoid observer bias.

**Time-Lapse Video Microphotography:** Cells were plated in normal complete medium at 10^5 cells per 35-mm tissue culture dish. Cells were examined in a Zeiss RA standard microscope equipped with a thermostatically controlled chamber (YS-1 Telethermometer) and water immersion lenses. Cell activity was recorded with an RCA low light level camera on a Panasonic video tape recorder and observed on a Panasonic television monitor (36). Time of mitosis was recorded as the time from initial rounding up of cells to the completion of cytokinesis.

**RESULTS**

**α- and β-Tubulin Mutants are Temperature Sensitive**

Cmd-4 (β-tubulin mutant), Grs-2 (β-tubulin mutant), and Tax-1 (α-tubulin mutant) grow normally at 37°C but are all temperature sensitive for growth at 40.5°C as shown in Fig. 1, A and B. In this experiment all three mutants appear to have very similar extent of failure of cell growth at the nonpermissive temperature. The wild-type cells also showed some growth inhibition at the nonpermissive temperature but did go through several cell doublings, while the mutant cells doubled no more than once at 40.5°C. Fig. 2 shows that the relative cloning efficiency of the mutant cells was also quite reduced as compared with the wild-type cells after relatively short periods of incubation at 40.5°C. In this case, the Cmd-4 line showed the earliest loss of viability after incubation at the high temperature (16 h). At later times, the effect on cloning efficiency of Cmd-4 and Grs-2 appears to be very similar, while the effect on Tax-1 is less. We have previously performed a 4-h treatment with colcemid (final concentration, 0.05 μg/ml). Fusion of 5 x 10^5 mitotic CHO cells with 5 x 10^5 interphase cells, resulting in induction of prematurely condensed chromosomes (PCC), was performed with 200 hemagglutinating units of UV-irradiated Sendai virus in Tricine buffer (13). The mitotic index of the mitotic cell population was 99%. Air-dried slides were prepared by conventional techniques. 100 prematurely condensed chromosomes were screened for each strain. PCC spreads were scored and their position along G1, S, and G2 was determined (13, 14). Synthesis of DNA in S-phase prematurely condensed chromosomes was determined by autoradiography.
shown that all three mutant cell lines will not form visible colonies at 40.5°C after 7-d incubation, while the wild-type cells do, and have used this phenotype as the basis for selecting temperature-resistant revertants of Cmd-4 and Tax-1 (4, 5).

Mutants Have Higher Mitotic Index at the Nonpermissive Temperature

The mutant and wild-type cells were cultured at 37°C and shifted to 40.5°C for various periods to determine the effect of temperature on mitotic index. The wild-type cells at 37°C had a mitotic index of 1.7% which increased to 3.8% by 20 h of treatment at 40.5°C. All three mutant lines, Grs-2, Cmd-4 and Tax-1, started with a higher mitotic index at 37°C than the wild-type (3.4, 4.3, and 2.6%). This increased to 9.7% in Grs-2, to 8.6% in Cmd-4, and to 6.7% in Tax-1 by 20 h at 40.5°C. The rate of increase in mitotic index with increasing periods at the nonpermissive temperature was greater for all the mutants than for the wild-type cells, with Grs-2 cells showing the largest increase over time. Cells in all phases of mitosis were seen in mutant and wild-type cells with no specific stage of chromosome condensation being favored.

Mutant Cells Have Lengthened Duration of Mitosis at the Nonpermissive Temperature

The increased mitotic index may indicate that cells have a shorter generation time, or alternatively, that the duration of mitosis is increasing. This latter possibility was examined by time-lapse video microphotography at 40.5°C for periods of up to 35 h. The results indicate a striking difference between the wild-type and mutant cells at the nonpermissive temperature. As Table I illustrates, the time required for one complete mitosis remained fairly constant in the wild-type cells over a period of 20 h at 40.5°C. In contrast, Grs-2, Cmd-4, and Tax-1 cells showed an approximate doubling of the time needed for mitosis at the nonpermissive temperature. This increase in duration of mitosis supports our hypothesis that the increase in mitotic index seen in these cells at high temperature is not due to more frequent cell division, but to aberrations in the mitotic process causing a delay in mitosis.

In these time-lapse studies we also observed an occasional cell that would round up for mitosis and then flatten again without evidence of cytokinesis. (For example, 2 of 16 Grs-2 cells cultured for 20 h at 40.5°C failed to divide. Such cells were not included in Table I.)

Mutant Cells Are Multinucleated When Grown at the Nonpermissive Temperature

One possible consequence of the disturbance in mitosis and cytokinesis described above would be the eventual formation of multinucleated or micronucleated cells. As can be seen in Fig. 3, the number of multinucleated mutant cells increased dramatically after 45 h at the nonpermissive temperature. There was also some increase in the number of multinucleated cells in the wild-type cells, but there was a much greater increase in the mutant lines, Cmd-4, Grs-2, and Tax-1, with almost 100% of these cells showing multinucleation under these conditions. Cells were mostly binucleate at 24 h, with considerable variation in nuclear size and shape, and at 48 h became quite large and appeared to contain many small nuclei (micronucleation).

Mutant Cells Are Not Blocked at a Specific Stage in G1, S, or G2 at the Nonpermissive Temperature

To determine if the mutant cells were killed at high temperature because of a block in a particular portion of the cell cycle, we analyzed the stages in which these cells are blocked by premature chromosome condensation. Mitotic CHO cells were fused with unsynchronized wild-type or mutant cells grown at 37°C or 40.5°C for 24 or 48 h. After fusion, the chromosomes in the interphase cells are induced to condense

| Time at 40.5°C | Average duration of mitosis |
|---------------|-----------------------------|
|               | Wild-type | Cmd-4 | Grs-2 | Tax-1 |
| 0-5           | 23 (4)    | 16 (3) | 24 (3) | 24 (6) |
| 5-10          | 17 (5)    | 25 (3) | 45 (3) | 26 (4) |
| 10-15         | 14 (1)    | 29 (2) | 52 (3) | 33 (3) |
| 15-20         | 20 (2)    | 37 (2) | 53 (6) | 39 (6) |

Cells were examined by time-lapse video microphotography as described in Materials and Methods after being cultured at 40.5°C for various time periods. Numbers in parentheses indicate numbers of cells examined during each period.

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and their nuclear membrane disappears. The prematurely condensed chromosomes are analyzed under the microscope and their position along the cell cycle is determined (14, 23). Table II presents our findings. We have not found any stage of the cell cycle in which these mutants are specifically blocked. There were, however, minor changes in the numbers of cells found in particular cell cycle stages.

All the S-phase prematurely condensed chromosomes of cells incubated at 40.5°C for 24 h were found to be labeled. This was true for wild-type cells even after 48 h of incubation at 40.5°C. However, the mutant cells incubated at 40.5°C for 48 h behaved differently in this experiment since 40 to 60% of the S-phase prematurely condensed chromosomes were unlabeled indicating that these S-phase cells ceased to replicate DNA. Because of the small sample size of cells counted and the fact that the cells are poorly viable after 48 h, the significance of these minor changes in cell cycle stage and DNA replication after 48 h is not clear, but an absolute block in a specific stage of the cell cycle clearly does not occur.

Abnormal Mitotic Spindles in the Mutant Cells at the Nonpermissive Temperature

Previously, we have shown that the Cmd-4 cells have normal cytoplasmic microtubules at the nonpermissive temperature (5). This was also true for the Grs-2 and Tax-1 cells (unpublished data).

The structure of the mitotic spindles at the permissive and nonpermissive temperature was examined by immunofluorescence. After 24 h at 40.5°C, wild-type cells showed predominantly normal spindles as visualized by indirect staining with antitubulin. Grs-2 and Tax-1 also showed relatively normal spindles after this treatment. Cmd-4, on the other hand, showed many disrupted and abnormal spindles, along with normal spindles. After 48 h at 40.5°C, the wild-type cells again showed predominantly normal and some abnormal spindles, while all the mutant cell types showed predominantly abnormal spindles (Table III). At the high temperature, a common aberration in both mutant and wild-type cells was multipolar spindles (Fig. 4, d, f, and h). In many cases spindle figures at 40.5°C appeared fairly normal, but were less compact than normal spindles at 37°C and covered more area in the cell. Another common abnormality was a very disorganized spindle, with microtubules spread in a highly irregular pattern over a large part of the cell (Fig. 4, f and h). Whether these formations were derived from metaphase spindles or from an earlier or later stage of mitosis was not clear. In a small percentage of mutant cells, especially Cmd-4, we observed mitotic cells with very thick microtubules, possibly representing bundles of microtubules. Similar structures have been reported after treatment of wild-type cells with taxol (25). The Cmd-4 cells appeared the most sensitive to temperature, in terms of their spindle structure, followed by the Tax-1 cells and the Grs-2 cells. There was no one specific spindle abnormality that was consistently observed for any of the mutants.

DISCUSSION

Three CHO mutants previously shown to have distinct alterations in α-tubulin (Tax-1) and β-tubulin (Grs-2 and Cmd-4) have been shown to be temperature sensitive for growth and cloning ability. The studies reported here were undertaken to determine the underlying mechanism of the temperature sensitivity of these cell lines. All evidence points to a defect in the mitotic spindles at the nonpermissive temperature that causes abortive chromosome movements and failure of cytokinesis, resulting in large multinucleated cells and ultimately death. We cannot distinguish among the spindle defects present in the three independent mutants analyzed here. Since mutations in both α- and β-tubulin resulting in resistance to different drugs produce the same phenotype, it is possible that the same basic function of microtubules in spindle formation is affected in all the mutants.

On the basis of inhibitor studies, microtubules have been implicated in many cell processes: mitosis, saltatory motion, maintenance of cell shape, secretion, locomotion, and activity of cell surface receptors. We have not been able to document any alteration in these functions of interphase microtubules in the mutants. Cell shape and saltatory motion appear to be normal in Cmd-4 and Grs-2 at 40.5°C after 48 h, as observed by time-lapse video microphotography (unpublished data of the authors). Locomotion of CHO cells cannot be studied since wild-type cells are not motile, and secretion and function of cell surface receptors for prostaglandin E, seem to be grossly normal (unpublished data of the authors). The cytoplasmic microtubules also appear to be normal in these cells at the nonpermissive temperature (5, and unpublished data). Electron microscope pictures of mitotic mutant cells at the nonpermissive temperature also show microtubules in spindles that appear grossly normal in structure (unpublished data). An alteration in the total number or length of the microtubules, however, cannot be excluded.
FIGURE 4 Indirect immunofluorescence localization of tubulin in wild-type and mutant mitotic cells at 37°C and 40.5°C. Cells were grown for 48 h at 37°C or 40.5°C and mitotic cells were collected by shake-off and concentrated on a slide with a cytocentrifuge. Cells were treated and fixed as described in Materials and Methods and stained with rat anti-α-tubulin and rabbit anti-rat globulin labeled with rhodamine. Left figures: 37°C, (a) wild type, (c) Grs-2, (e) Cmd-4, and (g) Tax-1; right figures: 40.5°C, (b) wild-type, (d) Grs-2, (f) Cmd-4, and (h) Tax-1. Bar, 10 μM. a, c, e, g, and h x 390; b and f x 625.
Why do these mutants show defects only in their spindles, and not in the other microtubule-associated functions? There are several possibilities. One is that there are other defects, but our methods are not sensitive enough to detect them. Assuming this it not the case, another possibility is that our mutations all affect a class of α- and β-tubulins that function specifically in mitosis. Evidence is accumulating for the existence of multiple genes for both α- and β-tubulin in several organisms, such as in humans (8, 35), chickens (7), Drosophila (24), Chlamydomonas (27) and sea urchins (1). Whether all these different genes have different functions is unclear. In the case of Drosophila, one β-tubulin gene (17) has been identified as being specific for sperm cells; however, it appears to be involved in diverse functions such as nuclear shaping, assembly of the axoneme, and meiosis (18). We have preliminary evidence based on Southern blots of CHO DNA that confirms the presence of multiple genes for tubulin in CHO cells, but have no evidence for the expression of these genes or for specific tubulins being involved in, for example, cytoplasmic versus spindle microtubules. If there are different molecules involved, they may be very closely cross-reactive since our immunofluorescence data show that monoclonal anti-α-tubulin will bind both to cytoplasmic and spindle microtubules.

A third possibility which we favor is that the same tubulin genes are used for both cytoplasmic and spindle microtubules, but that the requirements for formation and functioning of the mitotic spindle from microtubules are more stringent than requirements for cytoplasmic microtubules. If this is true, it is possible that the mutants carry an alteration in the tubulin molecules that changes binding affinity for other tubulin molecules or microtubule-associated proteins. This alteration could be compatible with formation of normal cytoplasmic microtubules but might interfere with the more complicated process of spindle formation from microtubules and the progression of the spindle through the various mitotic stages. In other words, the same proteins might be involved but the tolerances for some “mismatching” in the proteins causing changes in binding affinities might be critical in the spindle, but not elsewhere. Since the processes of depolymerization and polymerization appear to be of paramount importance in spindle function, any change in these by altering the affinities of tubulins might prove deleterious. However, it is difficult to imagine how the very same proteins in one environment (interphase cell) can behave differently from those in another environment (mitotic cell) without proposing the interaction of other molecules to confer some specificity.

It seems likely that specific microtubule-associated proteins that bind tubulin are involved in spindle function, and that these are not involved in functioning of cytoplasmic microtubules. The alterations of our mutants might change this binding, thus disturbing normal spindle functioning, while not affecting the functions of cytoplasmic microtubules. We have no evidence for such spindle-specific proteins in CHO, although there have been reports of spindle-specific proteins in mammalian cells (15, 21, 37). There are also reports of localization of calmodulin (34) and cyclic nucleotide dependent kinase (2) to the mitotic spindle. We are hopeful that studies of some of the tubulin revertants (5) will reveal that some are due to suppressor mutations in genes coding for microtubule-associated proteins. The study of these mutants should enable us to determine if any mitosis-specific microtubule-associated proteins indeed exist.

What are the consequences of the mitotic defect in the mutants? Spindle formation and the spindle itself may be very sensitive to an increase in temperature, even in normal cells. The wild-type CHO cells responded in a similar manner to the stress of high temperature as did the mutant cells, although in a less pronounced fashion. The wild-type cells showed an increase in their mitotic index at the nonpermissive temperature and an increase in multinucleated cells. They also showed a decrease in their growth rate at the high temperature. While all these effects were much less pronounced than those in any of the tubulin mutants, they still may indicate the basic temperature lability of the spindle. The tubulin defects may simply exaggerate this lability by altering proteins. Time-lapse studies showed that between 5 and 10 h at the nonpermissive temperature the duration of mitosis increased in the mutant cells compared with no increase in the wild-type cells. After this period of time at the high temperature, some of the mutant cells appear to undergo abortive cell division, with a failure of cytokinesis. This increase in time of mitosis probably accounts for the increase in mitotic figures seen in the mutant lines after 20 h at the nonpermissive temperature. PCC data showed no block in any specific interphase stage in the cell cycle of these mutants. The mutant cells at the nonpermissive temperature apparently continue to cycle and are not specifically and completely blocked at metaphase. Cabral (3) has isolated a taxol-requiring CHO mutant, in which the molecular defect has not yet been defined, that has a somewhat similar phenotype when taxol is removed; the cells continue to go through the cell cycle, but spindle assembly is blocked and they became multinucleated, and eventually die.

While we now know that these cells are defective in mitosis at the nonpermissive temperature, the biochemical change in the functioning of the spindle is not yet apparent. We are hopeful that the combination of analysis of temperature-resistant revertants of these mutants that may have alterations in microtubule-associated proteins, and analysis of the number of active tubulin genes through molecular cloning will enable us to address this question.

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