The Effect of Heat Shock on the Cell Cycle Regulation of Tubulin Expression in *Physarum polycephalum*

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**ABSTRACT** In the myxomycete *Physarum polycephalum*, tubulin synthesis is subject to mitotic cycle control. Virtually all tubulin synthesis is limited to a 2-h period immediately preceding mitosis, and the peak of tubulin protein synthesis is accompanied by a parallel increase in the level of tubulin mRNA. The mechanism by which the accumulation of tubulin mRNA is turned on and off is not clear. To probe the relationship between tubulin regulation and cell cycle controls, we have used heat shocks to delay mitosis and have followed the pattern of tubulin synthesis during these delays. Two peaks of tubulin synthesis are observed after a heat shock. One occurs at a time when synthesis would have occurred without a heat shock, and a second peak immediately precedes the eventual delayed mitosis. These results are clearly due to altered cell cycle regulation. No mitotic activity is detected in delayed plasmodia at the time of the control mitosis, and tubulin behavior is shown to be clearly distinct from that of heat shock proteins. We believe that the tubulin family of proteins is subject to regulation by a thermolabile mitotic control mechanism but that once the cell has been committed to a round of tubulin synthesis the "tubulin clock" runs independently of the heat sensitive system. In delayed plasmodia, the second peak of synthesis may be turned on by a repeat of the commitment event.

The periodic synthesis of microtubular proteins during the naturally synchronous mitotic cycle of the myxomycete *Physarum polycephalum* offers an interesting model for studying the cell cycle regulation of gene activity. In its plasmodial phase, *Physarum* grows as a macroscopic syncytium that can contain billions of nuclei in a single, uncompartmented cytoplasm. As a consequence of this communal lifestyle, mitosis within each syncytium is naturally and almost perfectly synchronous. We have previously demonstrated that whereas most major proteins display no periodicity of synthesis during the plasmodial cell cycle, synthesis of the tubulin family shows a coordinate 20-40-fold increase in rate immediately before metaphase (6). In a plasmodium growing with an interval of 8-10 h between mitoses, the rate of tubulin synthesis begins gradually to increase ~2 h before mitosis, reaching its peak at prometaphase and rapidly returning to the basal level after mitosis. It was later demonstrated that the level of the tubulin mRNA's defines a similar peak (7).

At present the mechanism by which messenger level is modulated during the cell cycle is not clear. Studies of the effects of colchicine on tubulin synthesis in mammalian cells indicate that tubulin messenger levels are negatively regulated with respect to the unpolymerized tubulin protomer pool, yet there is no demonstrable difference in the rate of tubulin transcription assayed in nuclei isolated from colchicine-treated cells as compared with untreated controls (3). If cell cycle control does not act at the level of transcription of the tubulin genes, there remain myriad possible control points ranging from processing, polyadenylation, and transport of the primary transcript to stability of the mature mRNA.

Little is known about the relation of tubulin biosynthesis to the regulation of other events in the cell cycle. Intracellular microtubules (as visualized by indirect immunofluorescence) begin to appear 30 min before metaphase, well after the peak of tubulin synthesis begins; develop into a well-defined spindle; and disappear immediately after mitosis. No interphase microtubules are observed (5). It therefore seems unlikely that a drop in the protomer pool due to microtubule assembly is involved in the turning on of tubulin synthesis during the cell cycle. To begin to investigate these relationships, we have studied the effects of heat shock on the regulation of tubulin synthesis, with quite unexpected results.

When synchronous plasmodia growing at 26°C are briefly transferred to 37°C and then returned to 26°C, mitosis is delayed in a manner related to the timing of the heat shock relative to the mitotic cycle (1). As the timing of the heat shock approaches that of mitosis, the delay in mitosis tends to increase linearly. A possible interpretation is that the heat shock irreversibly inactivates a thermolabile protein needed for mitosis, effectively resetting the mitotic clock to an earlier...
protein accumulates. We have investigated the relationship between such delays and the timing and extent of tubulin synthesis. Our data indicate that two peaks of tubulin synthesis follow a heat-induced mitotic delay. The first peak coincides with that in an unshocked control plasmodium, and a second peak precedes the delayed mitosis. These results are consistent with the interpretation that the cell was committed to the first peak before the heat shock and that after the heat shock the commitment event was repeated, resulting in a second peak.

MATERIALS AND METHODS

Materials: All chemicals were reagent grade. Soy peptone was purchased from Gibco Laboratories (Grand Island, NY), yeast extract from Difco Laboratories Inc. (Detroit, MI), electrophoresis media from Bio-Rad Laboratories (Richmond, CA), and nucleases from Boehringer Mannheim Biochemicals (Indianapolis, IN).

Cultures: All plasmodia were of strain MCV. We maintained and plated cultures essentially as described before (6). In brief, plasmodia were maintained as microplasmodia in shaking culture at 26°C in simplified soy medium (6). Before an experiment, microplasmodia were concentrated by centrifugation, resuspended in water, and plated on a nitrocellulose filter (BA85; Schleicher & Schuell, Keene, NH) supported by a stainless steel grid over a nutrient agar lawn. After a 4-h incubation at 26°C the time of mitosis was determined by phase-contrast examination of ethanol-fixed biopsies. The second mitosis after plating usually occurred 11–13 h after plating.

Heat Shocking: For heat shock experiments, we cut 7-cm plasmodia and their nitrocellulose supports into two equal pieces at a time estimated to be 120–180 min before the second mitosis after plating. The control half was returned to 26°C, and the half to be shocked was transferred to a SSM plate prewarmed to 37°C. After 30 min at 37°C, the shocked plasmodium was returned to 26°C plate and incubated until mitosis was observed.

Protein and DNA Labeling: We performed protein pulse-labeling and analysis essentially as described by Burland et al. (2). In brief, 7-mm discs were cut out of the periphery of the growing plasmodium, overlaid with 125 μCi [35S]methionine (SI-235; Amersham Corp., Arlington Heights, IL), and incubated for 30 min. We then harvested the plasmodia and prepared them for two-dimensional electrophoresis. Aliquots containing 106 acid-insoluble counts per minute were analyzed by two-dimensional gel electrophoresis. Radioactivity was detected by fluorography after treatment with Fluorohance (Research Products International Corp., Mount Prospect, IL). Samples for continuous labeling studies were similarly prepared from plasmodia prelabeled with 20 μCi/ml [3H]thymidine (24066; International Chemical & Nuclear, Irvine, CA), and incubated at 26°C for 10 min. The plasmodia were then harvested in 0.25 ml 20 mM Tris-HCl, 0.1 M EDTA, 0.1 mg/ml protease K, 0.5% SDS, and incubated at 70°C for 15 min. To assay radioactivity, 0.02 ml of homogenate was added to 0.5 ml 0.5 N NaOH and hydrolyzed at 37°C for 4 h. The nucleic acid was precipitated by the addition of 0.5 ml 20% trichloroacetic acid, 0.5 N HCl, and the precipitate was collected by filtration through glass fiber filters (Whatman GF/C; Whatman Chemical Separation Inc., Clifton, NJ). Samples were solubilized in 0.2 ml TS-2 tissue solubilizer and counted in 7a30B cocktail (Research Products International Corp.).

RESULTS

Two Peaks of Tubulin Synthesis Follow a Heat Shock

In plasmodia of Physarum polycephalum, tubulin synthesis is under mitotic cycle control. The 40-fold increase in tubulin messenger levels that accompanies the premitotic peak in tubulin protein synthesis indicates that the controls act primarily at the RNA rather than at the translational level. Yet little is known about the integration of the signals that govern the accumulation of tubulin mRNA with other events in the mitotic cycle. We have used heat shocking to alter the normal progression of mitotic events to investigate the coupling of tubulin synthesis with mitosis. By transferring a plasmodium from the normal growth temperature (26°C) to a higher temperature (37°C) for a short period (10–30 min), then returning it to the lower temperature, we can introduce a delay in the time of mitosis. The difference between the time of mitosis in the control and the heat shocked plasmodium has been called excess delay. Within limits, excess delay increases as the heat shock is applied closer to the time of the control mitosis. This has been interpreted as the result of thermal inactivation of a mitotic timer that must accumulate to a threshold level (1). In a sense, this forcing of the cell to reaccumulate the proposed mitogen can be visualized as a resetting of the mitotic clock to an earlier point.

We have found that when plasmodia of strain MCV are shocked for 30 min at 37°C, mitosis is delayed for up to 3 h. For our purposes, the most illustrative delay is induced by a shock that begins 3 h before mitosis, resulting in a 2-h delay in mitosis; i.e., the delayed mitosis occurs 5 h after the heat shock. The pattern of tubulin pulse-labeling during a single representative experiment is shown in Fig. 1. There clearly are two peaks of tubulin synthesis. The first precedes the time of mitosis in the control; i.e., the time mitosis would have happened in the absence of heat shock. A second distinct peak precedes the delayed mitosis. The duration and the amplitude of both peaks are similar to those of the control. Seven similar experiments are summarized in Table I. In each case, the first peak precedes the control mitosis and the second peak precedes the delayed mitosis, irrespective of the timing of the heat shock or the length of the delay. Note that two of these experiments deviate from the expected relationship between the timing of the heat shock and the length of the delay, yet even in these cases both peaks occur at the proper times. These data indicate that the first peak of tubulin synthesis is not a delayed response to the heat shock but rather that it reflects a consequence of altered mitotic regulation.
FIGURE 1 Tubulin synthesis after heat shock. Half of a plasmodium grown at 26°C was transferred to 37°C for a 30-min period beginning 180 min before anaphase of the second mitosis after plating. The other half of the plasmodium was kept at 26°C as a control. At time intervals thereafter, 7-mm circles of plasmodium were labeled with 25 μCi [35S]methionine for 30 min and harvested. Samples containing 50,000 cpm of labeled protein were analyzed by two-dimensional electrophoresis, and the tubulin spots were detected by fluorography. The sample times represent the midpoint of the 30-min labeling period. A presents examples of fluorograms representing the most significant time points, and B presents a quantitative treatment of the same gels. The alpha-1 tubulin and actin spots were cut out of each gel and counted. Tubulin counts per minute (CPM) are normalized to 5,000 actin cpm. The mark labeled Mc indicates the time of the control mitosis, and the mark labeled Mhs indicates the time of mitosis in the heat shocked plasmodium. Note that both peaks of labeling are evident by visual inspection of the fluorograms.

TABLE 1
Two Peaks of Tubulin Synthesis Follow a Heat Shock

| Heat shock | Peak 1 | Peak 2 | Delayed mitosis |
|------------|-------|-------|-----------------|
| -45        | 0     | 175   | 180             |
| -60        | -30   | 100   | 125             |
| -90        | 0     | >60*  | 140             |
| -120       | -40   | >90*  | 130             |
| -125       | -35   | 115   | 135             |
| -145       | -10   | 170   | 185             |
| -220       | -20   | 90    | 110             |

The data from seven independent experiments are summarized above. In each case, two peaks of tubulin synthesis were observed. The times indicated mark the beginning of a 30-min heat shock, the maximum of each peak, and the time of mitosis in each heat shocked plasmodium relative to the time of mitosis in unshocked controls. Note that the first peak always precedes the control mitosis and that the second peak always precedes the delayed mitosis, irrespective of the time of the heat shock. As presented, the time of the delayed mitosis corresponds to the mitotic delay. Times marked with an asterisk represent the last point taken before mitosis.

A Peak of DNA Replication Does Not Follow the Initial Peak of Tubulin Expression

One possibility to be considered was that a subpopulation of nuclei that had somehow escaped the mitotic delay were dividing at the time of the control mitosis and they were responsible for triggering the first peak of tubulin synthesis. Although microscopic examination of nuclei in the heat-shocked plasmodium during the control mitosis revealed a homogenous population of interphase nuclei, it was still conceivable that some dividing nuclei had escaped observation. If so, there ought to be an increase in DNA replication after mitosis in the escaped nuclei. As the data presented in Fig. 2 indicate, there is no increase in DNA labeling correlated with S-phase in the control. Cell fusion studies have indicated that replication continues autonomously in S-phase nuclei introduced into a G2 cytoplasm (4).

Tubulins Are Not Heat Shock Proteins

It was also possible that the tubulins behave as heat shock proteins whose synthesis is directly stimulated by the heat shock. If this were true, then tubulin synthesis should follow a heat shock irrespective of when during the mitotic cycle the shock is applied. To examine this possibility, a series of plasmodia were shocked 1-3 h after mitosis, well before the normal time of tubulin synthesis. In each case, a family of heat shock proteins (Fig. 3) showed an immediate rise in synthesis after the heat shock, but none of the tubulins was included in this family. The data shown in Fig. 3 C contrast the synthesis of two heat shock proteins with that of alpha-1 tubulin.
Effect of Heat Shock on Tubulin Levels

A possibility that has been suggested is that the hypothetical mitogen is actually one of the microtubular proteins. Were this the case, the pool of this protein might be irreversibly inactivated by the heat shock, and more would need to be made. The first peak of synthesis might restore the initial level of mitogen, but a second round of synthesis would be needed to boost the pool to the higher level needed to trigger mitosis. Using two-dimensional gel electrophoresis, we followed the levels of the tubulin pools in heat shocked and control plasmodia continuously labeled with $^{35}$S-methionine. The intensities of the tubulin spots at each time point were determined by scanning densitometry. The accumulation of alpha-1 tubulin is presented in Fig. 4. After the heat shock tubulin levels do not drop immediately, but rise as expected at the time of tubulin synthesis, then drop to a low level, and finally rise to a peak at the time of mitosis. As documented previously (6), there is a period of transient instability during the first 100 min after mitosis. The turnover is more pronounced in this study, resulting in a fivefold drop in tubulin level after mitosis. This difference may result from different strains having been used in the two studies. The former was done with CL, a haploid isolate, whereas the present study uses M2CV, a diploid isolate. However, it is quite clear that in addition to the turning on and off of the tubulin synthesis, the normally postmitotic period of tubulin turnover is also triggered in delayed plasmodia at the time of the control mitosis.

It is clear that tubulin levels in the heat-shocked plasmodia did not rise in two steps as might be expected. Rather, the tubulin newly made during the first peak of synthesis is turned over, and accumulation during the second peak never quite reaches that of the control. However, in both the delayed and control plasmodia the tubulin level reaches a maximum immediately before mitosis. Perhaps spindle assembly depends on the increasing of the protomer concentration beyond a critical threshold level. Although our data clearly rule out the possibility that extensive tubulin turnover results from the heat shock, they fail to exclude the possibility that the hypothetical mitogen is a microtubular protein.

DISCUSSION

The period of tubulin synthesis during the plasmodial mitotic cycle defines at least two events: the start and the end of the synthetic period. The initiation of the synthetic period clearly must be gated as a periodic cell cycle event, whereas termination of the period might not be under active cell cycle control. The rapidity and the precise timing by which the period is ended argue that the end, as well as the beginning, of the tubulin peak is under some active mode of cell cycle regulation. When this study was initiated, we anticipated that heat shocking would to some extent dissociate the two events by prolonging the period of synthesis. This clearly would be the case if termination depended on the completion of a mitotic event, e.g., spindle assembly. Then one would expect that initiation might either be delayed or unaffected by the heat shock and that termination would follow the delayed mitosis. Our results clearly rule out this hypothesis. The fact that the first peak of tubulin synthesis always coincides with the control mitosis and the second peak always coincides with the delayed mitosis supports the interpretation that termination of tubulin synthesis does not depend on completion of a major mitotic event. In the delayed plasmodium, there is no evidence of mitotic activity that can be correlated with the
FIGURE 4 Variations in tubulin level followed by a continuous label. Proteins were labeled by the addition 20 μCi/ml [35S]methionine to the medium at the time plasmodia were plated. Approximately 160 min before mitosis, half of the plasmodium was heat shocked and the remaining half was kept at 26°C as a control. After the heat shock, the delayed plasmodium was returned to the same plate as the control. Discs (7 mm) were harvested at different time points and analyzed by two-dimensional electrophoresis. Radioactivity was detected fluorographically. Prefogged films were developed after a 30-d exposure, and spot intensities were quantitated by scanning densitometry. A presents representative fluorograms and B presents the results of the quantitation. Since the action spots were overexposed, three other constant proteins, X (40,000, 5.72), Y (45,000, 5.72), and Z (56,000, 4.90), were used as internal standards. The alpha-1 tubulin levels are in arbitrary units from integration of background-subtracted densitometer tracings normalized to the sum of the areas under the X, Y, and Z tracings. They are useful only for purposes of comparison.

first peak. Therefore, termination may well be subject to regulation by a mitotic timing mechanism rather than be strictly dependent on the completion of mitosis.

Since the timing, amplitude, and duration of the first peak are equivalent to those of the control peak, it would seem that these parameters are not affected by the heat shock. Since the mitotic timer is strongly affected by thermal shock, it would follow that the tubulin timer to some extent does not depend on the mitotic timer. Our present working hypothesis is couched in the terminology of developmental biology. After a commitment event, tubulin synthesis is regulated by a heat shock-independent timing mechanism to coincide with mitosis. When a plasmodium is heat shocked after commitment, the timing of the first peak of tubulin synthesis has already been determined. The heat shock resets the thermo-labile mitotic timer to a point before commitment, and determination of a second round of synthesis happens as commitment is recapitulated, which results in the second peak before the delayed mitosis.

Analysis of the level of the tubulin pools after a heat shock answers some questions and raises others. It has been proposed that the mitotic timer involves the accumulation of a protein mitogen that triggers mitosis as a threshold is passed. Heat shocking denies the mitogen, resulting in a delay while more of the mitogen accumulates. Since the assembly of microtubules is heat sensitive, it has been suggested in light of their periodic synthesis that the mitogen is a microtubular protein. Were a microtubular protein denatured by a heat shock, it would probably soon be degraded. It might then require one burst of synthesis to replenish the pool and a second round to reach the threshold level. It is clear that the heat shock does not result in the wholesale turnover of any of the preexisting tubulin pools in the 100 min after the shock. Therefore, if a tubulin is the mitogen, the denatured form is not rapidly degraded. However, there does not appear to be a doubling in the tubulin pool level in the heat shocked plasmodium as compared with the control. Rather, the newly made tubulin synthesized during the first peak is turned over, perhaps because it is not protected in a microtubular structure, but probably because the turnover involves an active mechanism. Otherwise, it is quite difficult to explain why in delayed plasmodia, tubulins that are stable during most of interphase become unstable after the time of the control mitosis. In our original study, a period of tubulin instability was evident for a short period after mitosis (6). It is possible that the same mechanism responsible for postmitotic turnover is activated after the first peak of tubulin synthesis. Were this the case, then three tubulin-related events would be dissociated from mitosis by the heat shock: the turning on of synthesis, the turning off of synthesis, and the transient increase in tubulin instability.

Wright and Tollon (8) have observed a similar double peak of thymidine kinase activity in Physarum after a heat shock, rather than a single peak in controls. In the case of thymidine kinase, activity has been shown to increase before mitosis and remain high through S-phase, which immediately follows mitosis. However, interpretation of their results was complicated by the fact that enzyme activity is not necessarily related directly to protein synthesis. In light of our results, their conclusions gain considerable credibility. It may well turn out that the regulation we have observed relates not only to microtubular function but also to a wider family of products produced in anticipation of mitosis.

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