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Tubulin Expression and the Cell Cycle of the *Physarum* Plasmodium

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The high mitotic synchrony and macroscopic size of the *Physarum* plasmodium present a favorable opportunity to study molecular events in an unperturbed eukaryotic cell cycle. This proliferative form displays several biological features that we must appreciate at the outset:

1. As a syncytium, the plasmodium employs closed nuclear mitoses. The spindle is nucleated within the nucleoplasm just prior to prophase. The nuclear membrane seems to remain intact throughout mitosis, with the possible exception of localized disruption near the poles in telophase (Havercroft and Gull 1983).
2. No microtubules can be found in the plasmodial cytoplasm (Havercroft and Gull 1983). Thus, tubulin is used only in the spindle and is a minor component, representing on the order of 10^{-3} of total plasmodial protein (Laffler et al. 1981).
3. The plasmodium is distinguished by its natural system of somatic fusion (Poulter and Dee 1968) that permits rapid formation of heterokaryons between genetically compatible partners that differ in mitotic schedule and/or in size. Analysis of such fusions has indicated that nuclear division is controlled by a cytoplasmic condition that can be averaged between partners (Rusch et al. 1966; Tyson 1982).
4. There is no G_1 phase in the cell cycle of the plasmodium, just as in the rapid cleavage cycles of early embryos (Blumenthal et al. 1974; Harland and Laskey 1980). In a total cycle of 10–12 hours, S phase occupies 2–3 hours (Kubbies and Pierron 1983), G_2 8–9 hours, and mitosis 30 minutes.

5. Key events that coordinate the plasmodial cell cycle seem to lie immediately before mitosis rather than after. Mitotic averaging in heterokaryons (see above) operates until a point of nuclear commitment arises in late G_2 phase (Loidl and Sachsenmaier 1982; Tyson 1982). Furthermore, mutants arrested before metaphase show that reentry into DNA replication is not dependent on nuclear division (Laffler et al. 1979; Burland and Dee 1980).

DISCUSSION

We have investigated whether any of the macromolecules that participate in cell-cycle events are preferentially synthesized at the stage when they are used. The tubulins of the mitotic spindle have drawn our initial attention. Several experimental advances have been crucial in this work: (1) development of conditions for microtubule assembly in vitro from both myxamebal and plasmodial tubulins, permitting unambiguous identification of these molecules (Roobol et al. 1980, 1983); (2) development of conditions for growing plasmodia up to 30 cm in diameter (the "preparative plasmodium"), permitting one to isolate multiple samples for biochemical and cytological analysis over the cell cycle of a single plasmodium (Schedl et al. 1984); and (3) isolation conditions for plasmodial nuclei that retain the organization of microtubules (Roobol et al. 1984).

Cytological Analysis of Mitosis

Cytological analysis of the plasmodial cell cycle has been carried out both by indirect immunofluorescence, utilizing polyclonal and monoclonal anti-tubulin antibodies (Havercroft and Gull 1983; E.C.A. Paul and K.E. Foster, unpubl.), and by electron microscopy of thin sections (Schedl et al. 1984). One major conclusion of this work is that no organized tubulin polymer can be detected in the plasmodium outside of the mitotic interval beginning 30–60 minutes before metaphase and ending 30 minutes after metaphase in the 10–12-hour cell cycle. A second conclusion is that microtubules start to be organized within the nucleoplasm 30–60 minutes before metaphase. A bipolar spindle then matures at metaphase. In Figure 1, immunofluorescence of a smear from a metaphase plasmodium illustrates the high synchrony and intranuclear nature of the mitotic spindles. Finally, the intranuclear spindle is disassembled after division, with short microtubules detectable at 15 minutes after metaphase and none at 30 minutes. In summary, the plasmodium displays microtubules only within the nucleus and only within the mitotic period, 30–60 minutes before metaphase to 15–30 minutes after.

The earliest time at which microtubules can be detected corresponds well to the time discussed above at which plasmodial nuclei seem to be committed to proceed through mitosis when introduced by fusion into a lagging partner.

Premitotic Labeling of Spindle Tubulins

Over the past several years, we have investigated whether the tubulins for the mitotic spindles of the plasmodial nuclei are selectively synthesized during mi-

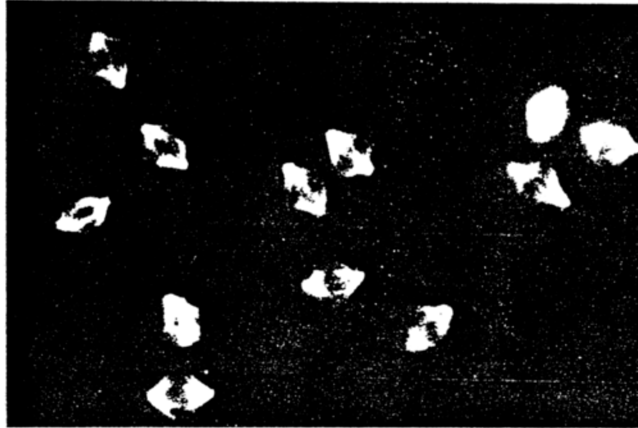

Figure 1
Spindles of synchronous metaphase nuclei. Nuclei isolated from a metaphase plasmodium were stained for indirect immunofluorescence. The primary antibody was the monoclonal anti-β-tubulin DM1B (Blose et al. 1984).

tosis. These experiments were initiated by T.G. Laffler and M. Polanshek (University of Wisconsin, Madison) and, independently, by G. Turnock (University of Leicester, United Kingdom). An account of this series of studies is given by Schedl (1984) and by Schedl et al. (1984). In this paper, we excerpt the points salient to our understanding of tubulin expression in the plasmodial cell cycle.

When plasmodia are pulse-labeled with [^{35}S]methionine for different 15-minute intervals in the 10-hour cell cycle and the labeled polypeptides are then resolved on two-dimensional gels and detected by fluorography, the plasmodial tubulins are unusual in showing periodic labeling. They are labeled effectively during late G_2 phase of the cell cycle but not after mitosis (Fig. 2, a vs. b). Pulse-chase and double-label control experiments have shown that the selective premitotic labeling cannot be explained by selective recovery at mitosis. Labeled tubulin polypeptides are metabolically stable and can be detected at all times in the cell cycle by silver staining the electrophoretograms from plasmodial lysates (Fig. 2, c vs. d).

The rise and fall in tubulin synthesis seen in vivo can also be demonstrated by translation in vitro. When total plasmodial RNA is extracted at different times in the cell cycle and then translated in vitro, tubulins are produced by premitotic, not postmitotic, RNA.

Multiple tubulin isotypes are expressed in the plasmodium and are found in isolated metaphase plasmodial nuclei (Burland et al. 1983; Roobol et al. 1984 and this volume). Is the selective premitotic synthesis of tubulins coordinate over this set of isotypes? The synthesis of all isoforms rises and then decreases around metaphase; however, the plasmodium-specific β_2 isotype seems to be turned off earlier than the other tubulins, as judged from labeling in vivo or from translation in vitro (Schedl 1984; Schedl et al. 1984). The newly synthesized tubulins are able to participate in the spindle; molecules radiolabeled in G_2 phase are found in spindles isolated from the next metaphase (E.C.A. Paul and A. Roobol, unpubl.).

Figure 2
Pulse-labeled and total tubulin polypeptides before and after plasmodial mitosis. Plasmodia were labeled for 15 min with [^{35}S]methionine, beginning either 60 min before (−60) or 60 min after (+60) metaphase. Proteins were resolved on two-dimensional gels as described by Burland et al. (1983). Pulse-labeled polypeptides were detected by fluorography, and total polypeptides by silver staining of parallel gels (Wray et al. 1981). (*a*) Fluorograph of −60 sample; (*b*) fluorograph of +60 sample; (*c*) silver stain of −60 sample; (*d*) silver stain of +60 sample. A indicates the position of actin. Spot Z is not a tubulin (Burland et al. 1983).

Kinetics of the Rise and Fall of α-Tubulin RNA Level

Quantitative studies of the kinetics of tubulin gene expression over the cell cycle have become feasible with the isolation of a *Physarum* cDNA clone for α-tubulin, templated by premitotic plasmodial RNA (Schedl et al. 1984). It has been possible from a single preparative plasmodium to extract sufficient RNA for quantitation at a series of time points in the cell cycle and also to assess cytologically the state of organization of the mitotic spindle microtubules. Levels of α-tubulin RNA were measured by dot-blot hybridizations in which a radiolabeled α-tubulin cDNA probe was hybridized to total plasmodial RNA (Schedl et al. 1984). The changes in α-tubulin RNA levels fit very closely to exponential kinetics, with a doubling time of 80 minutes for the rise and a half-life of 20 minutes for the fall. The amplitude of modulation is at least 40-fold (Fig. 3).

The very high synchrony of the plasmodium, both in S phase (Kubbies and Pierron 1983) and in mitosis (Schedl et al. 1984), makes it unlikely that asynchrony contributes significantly to the exponential character of this rise and fall. It seems more likely that the apparently exponential behavior is an intrinsic feature of the processes whereby levels of tubulin RNA are increased and then decreased.

These kinetics of modulation in tubulin RNA level can be explained by any of a broad range of models. Changes in rate of synthesis or in rate of turnover can

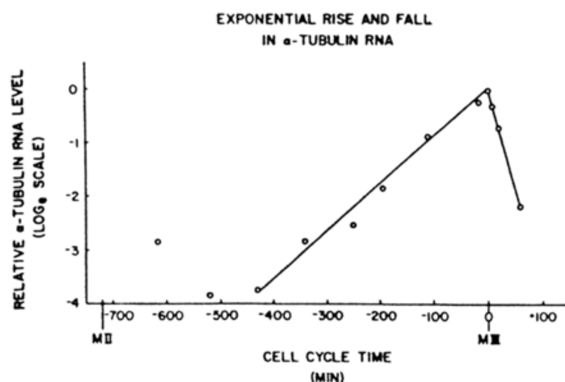

Figure 3
Relative α-tubulin RNA level over the plasmodial cell cycle. Total RNA samples were extracted from segments of one preparative plasmodium at different times in the cell cycle. Progress and synchrony of the cycle were assessed by phase and electron microscopy of the same plasmodium. The level of RNAs homologous to probes for α-tubulin, actin, and *Physarum* cDNA clones 16 and 42 were measured by quantitative dot-blot hybridization. The relative level of α-tubulin RNA was calculated by normalizing to the level of actin, clone 16, or clone 42; each normalizing sequence gave the same result (cf. Schedl et al. 1984).

be invoked. Here, we assess one plausible model for tubulin RNA accumulation over the cell cycle. In this model, the transcription of tubulin genes would be switched on to a constant rate in G_2 (zero-order synthesis) and then switched off at metaphase. Tubulin RNA would have a constant half-life, so that after metaphase, levels would fall by first-order kinetics. In such a model, RNA would accumulate asymptotically toward a plateau prior to mitosis and then fall exponentially after mitosis. The data shown in Figure 3 are quantitatively at odds with such a model, because the accumulation curve is apparently exponential. Thus, we seriously consider that the G_2 phase synthesis of tubulin RNA involves an accelerative component, e.g., an autocatalytic one. Below, we suggest one possible mechanism out of many with this feature.

The fall in α-tubulin RNA after metaphase is very rapid. The upper limit of the postmitotic half-life is 20 minutes, corresponding to only 3% of the cell-cycle time. Here, we consider the possibility of a facilitated decay. Established examples of regulation by facilitated decay include yeast histone RNAs (Hereford et al. 1981; Osley and Hereford 1982) and adenoviral early RNAs (Babich and Nevins 1981). Again, we return below to one example of such a process.

Cell-cycle Modulation of Tubulin RNA and Autoregulation

Two decades ago, it was suggested that gene activity might fluctuate over the cell cycle in response to a negative feedback control exerted by metabolites created by cell-cycle activity (see Mitchison 1969). This formalism, oscillatory repression, might be adapted to explain the rise and fall in tubulin gene expression over the plasmodial cell cycle. Indeed, many animal cell lines seem to display a negative feedback response, wherein tubulin RNA levels fall when the

levels of tubulin protomer are increased, either by treatment with the microtubule-depolymerizing agent Colcemid or by microinjection of tubulin (Ben-Ze'ev et al. 1979; Cleveland et al. 1981, 1983). For the *Physarum* plasmodium, the time course of the fall in α-tubulin RNA is coincident with the depolymerization of microtubules, and so is concordant with negative feedback by protomer. In contrast, the time course of the rise in α-tubulin RNA is not directly consistent with release from negative autoregulation by tubulin protomer. As summarized above, tubulin polymerization is initiated only in the interval between 30 and 60 minutes before metaphase. But α-tubulin RNA levels rise perceptibly as early as 6 hours before metaphase, long before polymerization into microtubules would be extensive enough to deplete the total pool of protomer. Thus, negative autoregulation by tubulin protomer over tubulin gene expression is a possible explanation for the postmitotic fall in plasmodial α-tubulin RNA. But the initiation of the premitotic rise does not seem to be caused by a release from negative autoregulation by tubulin protomer.

Temporal Restriction of Macromolecular Synthesis

What is the significance of the restriction of tubulin synthesis to the G_2 phase of the plasmodial cell cycle? This restriction is particularly puzzling because the tubulin polypeptides seem to have a long half-life (see Fig. 2). Thus, it is not obvious how restriction in time of synthesis can lead to restriction in time of action, as occurs with the homothallism endonuclease of *Saccharomyces cerevisiae*, which is thought to be unstable (Nasmyth 1983).

Perhaps there is a cell-cycle restriction in the localization of the plasmodial tubulins. For the *Physarum* plasmodium, an appropriate model states that tubulins are imported into the nucleus during G_2 phase and then are exported after mitosis. Indeed, tubulin molecules radiolabeled in G_2 phase can be found in nuclei isolated from metaphase plasmodia but not those isolated from interphase (A. Roobol, E.C.A. Paul, and K.E. Foster, unpubl.). Since the plasmodial mitoses are closed, some mechanism of import is necessary (cf. De Robertis 1983). One might expect that a shuttling of proteins within the cell would be accompanied by reversible or irreversible modifications. There is no evidence, however, for a molecular modification of tubulin coincident with a cycle of nuclear import and export. It is crucial to recognize, also, that the buffers used for nuclear isolation contain detergent and may well not permit retention of protomeric tubulin.

An import/export model has a particularly simple intersection with the analysis above of the kinetic data in Figure 3. If there is an autocatalytic component to the synthesis in G_2, it may be served by imported nuclear tubulins, acting to increase nuclear tubulin RNA levels. On the other side of metaphase, facilitated decay of cytoplasmic RNA, if it exists, might be directed by tubulin molecules released from the nucleus after mitosis. This model, even if correct in outline, clearly needs further provisions to be complete. How is synthesis initiated in G_2? How would the facilitated decay be blocked within the nucleus and during the G_2 increase?

Numerous other models can be devised that would account for the exponential properties of the data in Figure 3. The one advanced here has the virtue of direct experimental test.

CONCLUSIONS AND PROSPECTS FOR DEEPER STUDY

Analysis of the synthesis of tubulins for the mitotic spindles of the plasmodium has shown that the pronounced modulation in synthesis reflects changes in levels of tubulin RNA. Tubulins synthesized in G_2 are utilized to construct the upcoming mitotic spindle. The rise and the fall each follow apparently exponential kinetics. The rise is initiated hours before the pool of protomer is depleted by microtubule formation, whereas the fall is contemporaneous with spindle disassembly after metaphase. The rapidity of the fall in tubulin RNA suggests a process of facilitated decay. One model to explain the exponential character of the rise in tubulin RNA states that tubulin molecules imported into the nucleus during synthesis potentiate further tubulin RNA synthesis. The cell-cycle regulation of tubulin gene expression in the plasmodium is, of course, not the only well-studied example of cyclic modulation of gene expression. However, there is a useful set of experimental challenges and opportunities available in *Physarum* by which to deepen our understanding. A number of issues need to be investigated:

1. What are the relative contributions of changes in rate of synthesis and rate of decay of tubulin RNA to the kinetics of α-tubulin RNA level given in Figure 3?
2. What are the patterns of entry and exit of tubulins from the nucleus? Can tubulin molecules used in the spindle in one cell cycle be re-used in subsequent cycles?
3. Are there any functional differences between premitotic and postmitotic tubulin polypeptides, involving structural differences too subtle to detect by our current methods?
4. Are there any true autoregulatory components to the modulation of tubulin gene expression in the plasmodial cell cycle, rigorously demonstrable by mutations lying within a tubulin structural gene (cf. Smith and Magasanik 1971)?
5. Is there a cell-cycle modulation of plasmodial spindle components other than tubulin?
6. What features of the tubulin modulation seen in the *Physarum* plasmodium are unique to the special biology of this form and what features are general to other eukaryotes?

Several laboratories are addressing these issues. On the molecular side, it is clearly essential to capitalize on the high synchrony of the plasmodium to obtain an unambiguous pulse-chase analysis of the rates of tubulin RNA synthesis and decay. It is clearly also important to master the injection of cytoplasmic fractions into plasmodia. Injections of volumes up to 1 μl have been reported (Adams et al. 1981) but are not yet routine due to the wounding and gelling reactions of the plasmodium. On the genetic side, the capability for selectional genetics in the uninucleate, haploid myxamebal phase of *Physarum* needs to be utilized to obtain mutants affected in the tubulin structural genes and their regulators.

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