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Periodic synthesis of microtubular proteins in the cell cycle of *Physarum*

(periodicity/synchrony/mitosis/tubulin/nuclear division)

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ABSTRACT **Periodic polypeptide labeling over the naturally synchronous nuclear replication cycle of *Physarum polycephalum* was analyzed by fluorography of two-dimensional electropherograms. Two sets of polypeptides, denoted as P and Q, showed strong periodicity; they were maximally labeled just prior to mitosis. This periodicity was shown to reflect synthesis rather than turnover or recovery. Both P and Q copolymerized with porcine microtubular proteins and displayed electrophoretic properties similar to those of porcine tubulins. The significance of the periodic synthesis of these microtubular proteins is discussed as a possible component within the chain of events that establishes the high mitotic synchrony of *Physarum* syncytia.**

The replicative cell cycle of eukaryotes is marked by major cellular events such as entry into DNA synthesis (S phase) and progression through the stages of mitosis. Do these cellular events reflect molecular periodicities in protein synthesis?

One can distinguish between division cycles that generate apparently equivalent daughter cells and those cycles that yield frankly distinct progeny. In the differentiative cell cycle of *Caulobacter crescentus*, the generation of a new cell type, the swarmer, is accompanied by strong periodicities in the synthesis of a number of polypeptides such as the flagellin needed for this new cell type (1).

The situation for proliferative systems with equational division is less clear. Experimental analysis in these systems is complicated by the problem of distinguishing between a natural, endogenous periodicity and an artifactual one induced by the synchronization procedure (e.g., see ref. 2). Two approaches have arisen to deal with this problem: (*i*) postsynchronization methods such as elutriation or mitotic detachment, to separate prelabeled cells into temporal classes; and (*ii*) natural synchrony in a syncytium large enough for biochemical analysis, such as that of the myxomycete *Physarum polycephalum* (see ref. 3).

The first approach has been used to analyze the periodicity of labeling of polypeptides in *Saccharomyces cerevisiae* (4) and HeLa cells (5). Among the polypeptides that can be identified in two-dimensional electropherograms from *Saccharomyces* or from HeLa, the amplitude of labeling never changes more than 3-fold.

We have adopted the second approach, using *Physarum*. The extreme natural synchrony might permit detection of periodicities that would be obscured by a loss of resolution in postsynchronization methods.

We report that *Physarum* polypeptides with properties of microtubular proteins are synthesized preferentially in the premitotic interval of the division cycle. The amplitude of the periodicity is at least 30-fold.

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MATERIALS AND METHODS

Cultures. The general culture methods have been described by Mittermayer *et al.* (6). Microplasmodia of strain CL (7) were grown in suspension culture in a simplified soy medium (SSM) adapted from a medium of E. Brewer and B. Prior (personal communication): per liter, 10 g of Difco BactoSoytone, 3 g of yeast extract, 9 g of glucose, 3.6 g of citric acid, and 0.5 g of $MgSO_4 \cdot 7H_2O$; adjusted to pH 4.6 with KOH; 0.1% hematin added after autoclaving. The substitution of soy peptone for tryptone reduces to one-quarter the methionine level of the medium. In dual-labeling experiments, the amino acid composition of the medium was further controlled by replacing soy peptone with a mixture of 50 mg of glycine, 270 mg of DL-alanine, 67 mg of L-arginine·HCl, and 28 mg of DL-methionine per liter (AAM; ref. 8).

Surface plasmodia were formed by the coalescence of microplasmodia grown in suspension culture (6). Synchronous mitoses MI, MII, and MIII are defined by the times of appearance of metaphase in each nucleus of a field viewed in smear preparations by phase-contrast optics (see ref.3).

Protein Labeling. For labeling experiments, a 0.2-ml inoculum of microplasmodia was plated on a nitrocellulose membrane (Millipore HAWP) supported by 2-mm glass beads in a 60-mm petri dish and fed with 4 ml of medium. SSM medium was supplemented with [^{35}S]methionine at 12.5–50 μCi/ml (New England Nuclear, NEG009T; 1 Ci = 3.7×10^{10} becquerels) for sulfur prelabeling experiments or with ^{3}H-labeled amino acid mixture at 80 μCi/ml (New England Nuclear, NET250) for tritium prelabeling experiments. The medium was renewed every 10 hr. For pulse-labeling, a 7-mm-diameter disc of plasmodium and membrane support was cut out with a cork borer. It was then incubated with 0.5–20 μCi of methionine in 5 μl of H_2O for 15–60 min.

Two-Dimensional Gel Electrophoresis. Samples were prepared for electrophoresis by harvesting plasmodial discs with 50 μl of ice-cold sonication buffer (10 mM Tris·HCl, pH 7.4/5 mM $CaCl_2$/10 mM $MgCl_2$ containing RNase at 50 μg/ml), immediately sonicated, and supplemented with DNase to 50 μg/ml. After incubation of the mixture for 5 min on ice, 50 μl of $NaDodSO_4$/lysine (50 mM lysine/0.2% $NaDodSO_4$), 90 mg of urea, and 150 μl of double-strength lysis buffer (9.5 M urea/10% 2-mercaptoethanol/4% Nonidet P-40/3% pH 5–7 plus 1% pH 3–10 LKB Ampholines) were added, and the extract was stored at −80°C.

Two-dimensional gel electrophoresis was carried out essentially as described by O'Farrell (9) and modified by Jackle (10)

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and Lowe *et al.* (11). Briefly, 3 × 10 mm tubular isoelectric focusing gels were run with 10 mM histidine as the cathodic buffer and 20 mM phosphoric acid/4% Nonidet P-40/9.5 M urea as the anodic buffer. The gels were stained after the first dimension (10). The appropriate bands were then cut out and run on a 1.5 × 140 × 180 mm 7.5% discontinuous $NaDodSO_4$ slab gel for the second dimension. These slab gels were stained, treated with EN^{3}HANCE (New England Nuclear), dried, and exposed to Kodak RP5 x-ray film at −80°C for 2–30 days.

The molecular weight markers were: 66,500, bovine serum albumin (Miles); 58,000, catalase (Sigma); and 41,500, wheat α-amylase (a generous gift from R. Boston).

Measurement of Radioactivity. The amount of label in material insoluble in 5% trichloroacetic acid was determined by diluting a 5-μl sample in 1 ml of H_2O, adding 10 μl of bovine serum albumin (1 mg/ml) as carrier, and acidifying with 50 μl of 100% (wt/vol) trichloroacetic acid. After 10 min on ice, the precipitate was collected on Whatman GF/C glass fiber filters, solubilized in 0.5 ml of Protosol (New England Nuclear), and assayed in 10 ml of scintillation cocktail (OCS, Amersham/Searle). Radioactivity in gels was quantified by cutting out the area of interest, solubilizing for 18–48 hr in 0.4 ml of 30% H_2O_2 and 0.2 ml of perchloric acid, and assaying in 10 ml of scintillation cocktail (Aquassure, New England Nuclear). Noncoincident counts were monitored, and counting efficiencies were determined by the external standard ratio method.

Copolymerization with Porcine Brain Microtubular Proteins. Microtubular polymerization/depolymerization conditions were those described by Borisy *et al.* (12) in which an increased temperature, H (30°C), promotes polymerization, and a decreased temperature, C (0–4°C), promotes depolymerization. The pellet and supernatant fractions at each step are denoted P and S respectively. A sample of purified porcine brain microtubular protein at the H_2P stage was kindly provided by Robert Scheele. Fresh PMG buffer (0.1 M piperazinethanesulfonic acid, pH 6.94/1 mM ethylene glycol bis(β-aminoethyl ether)*N,N,N′,N′*-tetraacetic acid/0.1 mM $MgCl_2$/1% Trasylol/0.1 mM GTP) was added to permit depolymerization on ice for 30 min. After centrifugation at 39,000 × *g* for 30 min at 4°C, the supernatant C_2S was taken for copolymerization.

A *Physarum* plasmodium (5 cm diameter) was labeled for 90 min prior to mitosis with 200 μCi of [^{35}S]methionine. It was then harvested in 5 vol of cold PMG buffer and sonicated. The 40,000 × *g* supernatant of this sonicate was used for copolymerization. This extract (protein, 5.9 mg/ml; 136,650 dpm/μl) was mixed with porcine brain microtubular protein (5.9 mg/ml) in a ratio of 1:40 by volume. This mixture was then taken through two complete cycles of polymerization and depolymerization, at 30°C and 4°C, respectively. After the first copolymerization step, a control series was carried out with 10 mM $CaCl_2$ present to inhibit microtubule assembly. Samples were removed at each step for determination of total protein. After addition of an equal volume of double-strength lysis buffer and of solid urea to 9.5 M, radiolabeled *Physarum* polypeptides were resolved by two-dimensional electrophoresis and detected by fluorography.

RESULTS

Periodic Polypeptide Labeling. Polypeptides labeled during a limited portion of the mitotic cycle were identified by comparing two-dimensional gel patterns of samples from plasmodia pulse-labeled with amino acids at different times in the cycle.

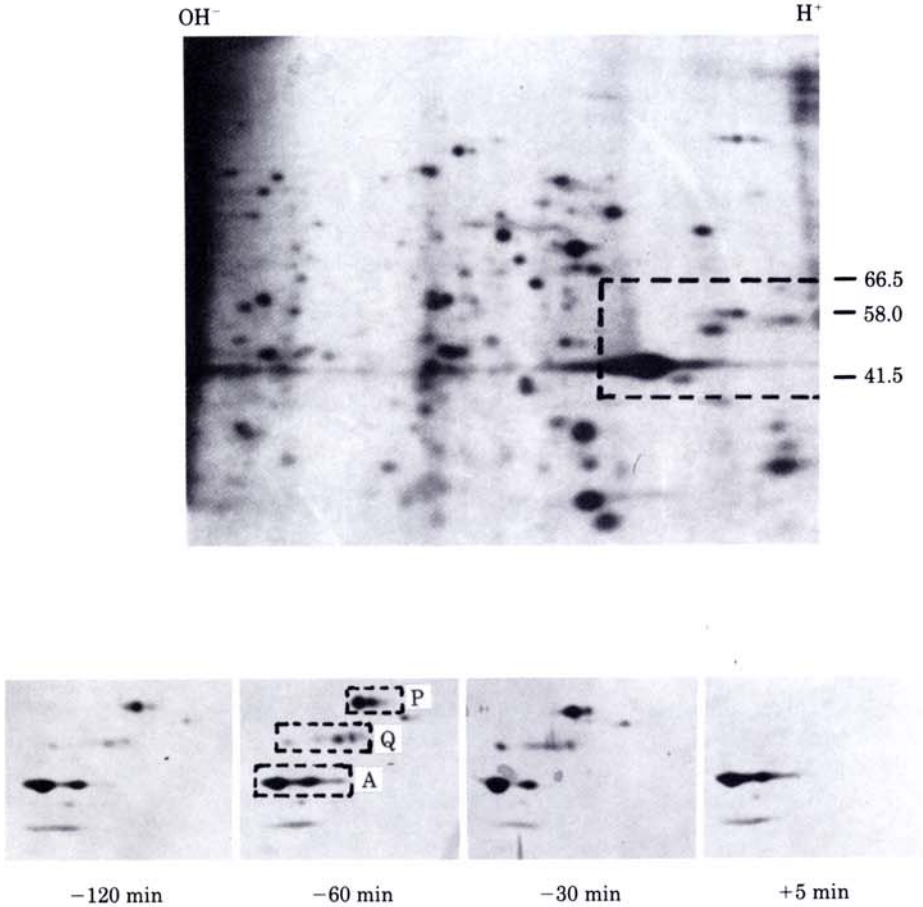

FIG. 1. Periodic polypeptide labeling. Discs (7 mm) cut from plasmodia were labeled with 5 μCi of [^{35}S]methionine in SSM for 30 min. Total protein (2×10^5 dpm/gel) was fractionated on two-dimensional O'Farrell gels and detected by fluorography. (*Upper*) Full array of species labeled late in G_2 phase. Isoelectric focusing was in the horizontal dimension and $NaDodSO_4$/polyacrylamide gel electrophoresis was in the vertical. Molecular weight markers are shown × 10^{-3}. (*Lower*) Detailed analysis of the area indicated by broken lines in *Upper*, showing presumptive actin (A) and microtubular proteins (P and Q) labeled starting at the indicated times relative to synchronous mitosis (nuclear division).

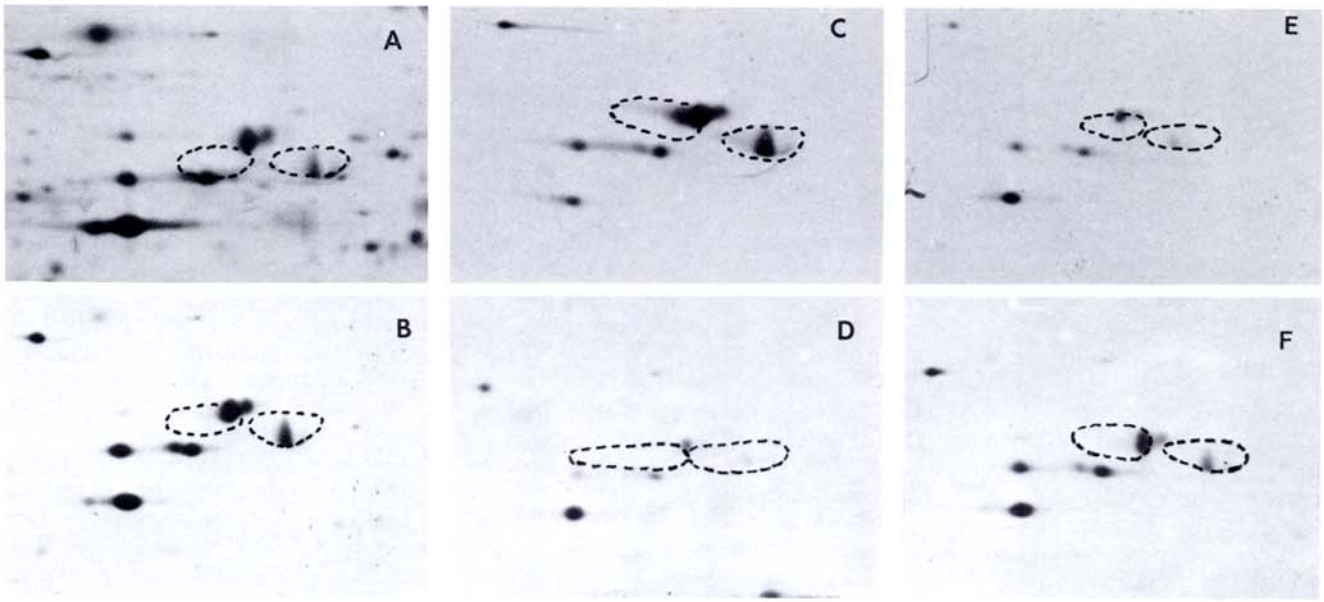

FIG. 2. Copolymerization of labeled *Physarum* polypeptides with porcine brain microtubular protein. A plasmodium was labeled late in G_2 phase. Protein in the extract was then mixed with carrier porcine brain microtubular protein and subjected to cycles of polymerization and depolymerization. Samples from the supernatant (S) or pellet (P) fractions for these steps were then resolved by two-dimensional gel electrophoresis, and labeled species were detected by fluorography; fluorograms were developed after 30 days of exposure at −80°C. Encircled zones correspond to the position of carrier porcine tubulin, as detected by staining. (*A*) Starting material (porcine-C_2S + *Physarum* sonicate), 100,000 dpm; (*B*) first-round copolymerization pellet (H_1P), 15,000 dpm; (*C*) second-round pellet (H_2P), 5000 dpm; (*D*) second-round copolymerization supernatant (H_2S), 7500 dpm; (*E*) calcium inhibition control pellet (H_2P, Ca^{2+}), 2500 dpm; (*F*) calcium inhibition control supernatant (H_2S, Ca^{2+}), 10,000 dpm.

Discs were cut from synchronous plasmodia at hourly intervals during the 10-hr mitotic cycle and were pulse-labeled with [^{35}S]methionine for 30 min. Polypeptides were resolved by two-dimensional gel electrophoresis, and label was detected by fluorography. Although most polypeptides were labeled uniformly throughout the cycle, several species in one region of the gel were labeled only during the premitotic phase. These polypeptides were labeled actively 3 hr before metaphase and maximally just prior to mitosis but only marginally after nuclear division (Fig. 1). These polypeptides have apparent molecular weights of 48,000 to 57,000 and isoelectric points between 5.1 and 5.4. In the small panels of Fig. 1, the intense set of spots to the lower left may be actin, designated A (molecular weight, 42,500; pI, 5.6). We have designated the higher molecular weight set of periodic polypeptides P and the lower molecular weight set, Q.

Copolymerization of Periodic Polypeptides with Porcine Microtubular Proteins. The molecular weights and isoelectric points of polypeptide sets P and Q resemble those of lower eukaryotic tubulins (13, 14). The possibility that these are microtubular proteins was explicitly investigated by determining whether any of the periodic polypeptides of *Physarum* were competent to polymerize with porcine microtubular protein. Labeled plasmodial proteins were taken through two polymerization/depolymerization cycles in the presence of carrier porcine protein. *Physarum* polypeptides from sets P and Q were enriched in the microtubular fraction (Fig. 2). When microtubule assembly was inhibited with 10 mM $CaCl_2$, the periodic *Physarum* species remained in the soluble fraction. The microtubular polypeptides P and Q did not comigrate precisely with porcine tubulins. It is known that the *Physarum* amoebal proteins capable of self-assembly do not comigrate with mammalian tubulins (ref. 15; unpublished data).

Kinetics of Labeling of Polypeptides P and Q. A detailed time course of the periodic labeling of sets P and Q was established by analyzing pulse-labeled material from the spots resolved as in Fig. 1. The periodic species were localized by autoradiography and then quantified. A correction for variation in sample loading and recovery was made by normalizing counts in P and Q to those in the presumptive actin spot, A. As documented below, labeling of A showed little variation over the mitotic cycle. The peak of periodic labeling of P and Q began at least 2 hr before metaphase, increased sharply until the time of mitosis, and then decreased precipitously (Fig. 3*A*). At the point of maximal labeling, just prior to mitosis, P and Q accounted for about 3% of the total radioactivity applied to the gel, second only to the presumptive actin species. These spots were barely detectable only 30 min later.

Is Periodic Labeling Due to Periodic Recovery? The observed increase in polypeptide labeling could be the result of changes in the rate of synthesis of these polypeptides, or it could be due to differential recovery or turnover of the microtubular proteins. The level of species P was followed during long-term continuous labeling. The relative level of species P increased during the latter part of G_2 phase but did not decrease dramatically after mitosis (Fig. 3*B*). Thus, there does not seem to be a strong periodicity in the recovery of prelabeled P.

To investigate explicitly the metabolic stability of polypeptide P, a plasmodium was prelabeled with [^{35}S]methionine for two generations and then transferred to unlabeled medium with excess methionine. There was little turnover of labeled P (Fig. 3*C*). The slow decline in the normalized activity of P after mitosis may reflect slow turnover of P or continued labeling of the normalizing spot A. If growth were balanced, the relative levels of two molecular species should not change between specific times in successive cycles. Thus, there does not seem to be a phase of rapid turnover to explain the periodic labeling of the microtubular polypeptides of *Physarum*.

Confirmation of Selective Synthesis by Dual Labeling. Plasmodia continuously labeled with amino acids containing ^{3}H were pulse-labeled with [^{35}S]methionine at hourly intervals throughout the mitotic cycle. A change in the rate of synthesis of a polypeptide would be reflected by a change in the ratio of

^{35}S to ^{3}H in the spot. The region presumed to represent *Physarum* actin, which accounted for 5% of the ^{3}H counts applied to the gel, showed no strong periodicity in the isotope ratio (Fig. 4*A*). By contrast, the microtubular polypeptide spots showed the same form and magnitude of periodicity in isotope ratio as found in the single-label analyses described above (Fig. 4*B*).

The constancy of the isotope ratio in the presumptive actin region justifies its use as a normalization standard in the single-label analyses. The strong periodicity in the isotope ratio of the microtubular spots confirms that these species are synthesized periodically. No artifact of gel loading or recovery seems to offer an alternative explanation.

DISCUSSION

In the synchronous mitotic cycle of *P. polycephalum*, we have found a family of polypeptides that is coordinately labeled in late G_2 phase (Fig. 1). These proteins, designated P and Q, have molecular weights similar to those of tubulins from lower eukaryotes (13, 14) including *Physarum* (15) and are capable of coassembly with porcine microtubular proteins (Fig. 2). The normalized level of these polypeptides, judged by continuous labeling, increases nearly 2-fold during late G_2 phase (Fig. 3*B*). Prelabeling experiments show that at least P is a largely stable species (Fig. 3*C*). The periodic labeling of the microtubular proteins is not displayed by other polypeptides in the actin region and cannot be attributed to changes in turnover or recovery, as shown by a double-label experiment (Fig. 4).

Strongly periodic protein synthesis in a proliferative eukaryotic cell cycle is not common. An instance of periodic polypeptide labeling in *Escherichia coli* has been reported by Gudas *et al.* (16).

Is the quantitation reliable? We minimized problems in quantitation by assaying gel spots directly. Only digestion in $HClO_4/H_2O_2$ was capable of solubilizing the dried, EN^{3}HANCE-treated gel. Solubilization was carried out for up to 48 hr to minimize chemiluminescence, and each sample was monitored for noncoincident counts. Because there is no labeling in the P/Q region immediately after mitosis, we conclude that contaminating proteins are not a severe problem.

Are we accounting for all of the P and Q polypeptide species? Proteolytic artifacts have been minimized by immediate solubilization of samples in Nonidet P-40/urea. Although the P polypeptide set is tightly grouped in the electropherogram, the Q set is heterogeneous. We have grouped together spots of different pI in quantitating the Q set.

Varying amounts of protein fail to enter the isoelectric focusing gel. We have controlled for the effects of such trapping by normalizing counts in P and Q to those in presumptive actin (Fig. 3) or by comparing short-term with long-term label (Fig. 4).

We do not exclude the possibility that the periodicity ob-

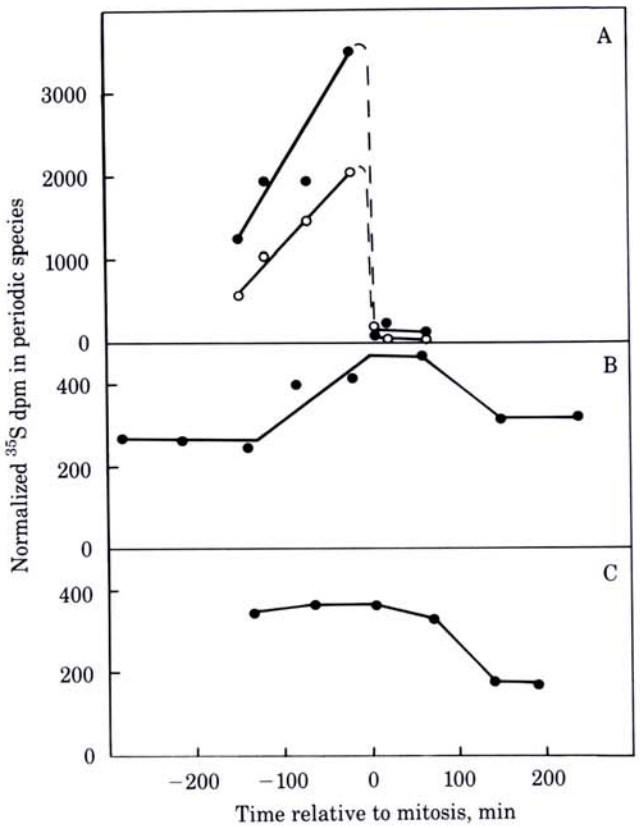

FIG. 3. Synthesis of *Physarum* microtubular polypeptides during the mitotic cycle. (*A*) Plasmodia were labeled as in Fig. 1, and polypeptides were analyzed on two-dimensional gels. Label in microtubular polypeptides was quantified by cutting out the appropriate regions of the dried gel, solubilizing, and assaying. Each point represents the midpoint of a 30-min pulse with 20 μCi of [^{35}S]methionine. ●, Polypeptide set P; ○, polypeptide set Q. Counts were normalized to 5000 dpm in presumptive actin. (*B*) A plasmodium was continuously labeled from the time of plating with 200 μCi of [^{35}S]methionine to follow the level of extractable microtubular polypeptides. (*C*) A plasmodium was prelabeled as in *B* with 200 μCi of [^{35}S]methionine and then transferred after mitosis II to unlabeled medium containing excess methionine. The level of prelabeled microtubular polypeptides in set P was assayed in extracts made before and after mitosis III.

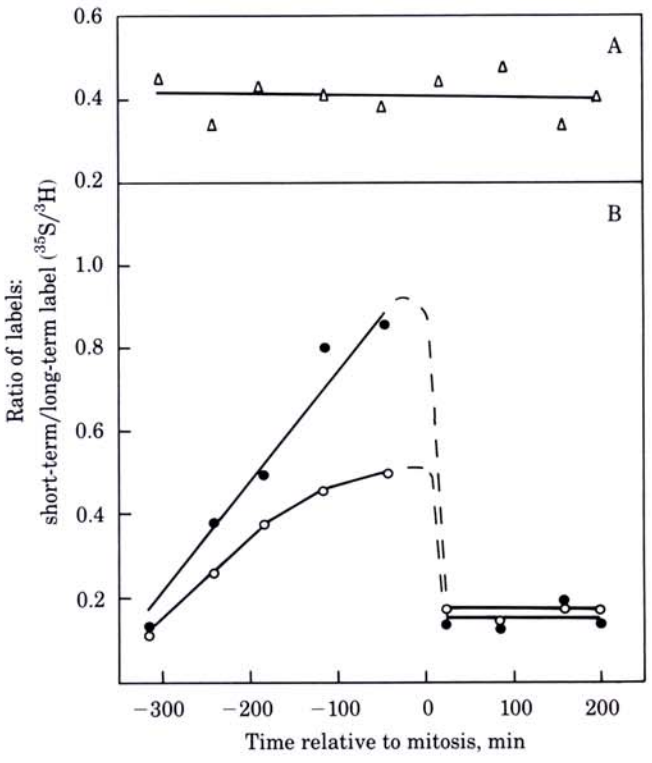

FIG. 4. Synthesis of microtubular polypeptides measured by isotope ratio. (*A*) A plasmodium was continuously labeled with 200 μCi of ^{3}H-containing amino acids in AAM, and then discs were pulse-labeled with 0.5 μCi of [^{35}S]methionine. The region of interest was cut out of two-dimensional gels, solubilized, and assayed. The ratio of the long-term ^{3}H label to the pulse ^{35}S label is plotted vs. time relative to mitosis II. (*B*) The microtubular polypeptide region from the same gels as above was similarly analyzed. ●, Polypeptide set P; ○, polypeptide set Q. Each point represents the midpoint of a 30-min pulse. Periodic synthesis of microtubular species is reflected by changes in the $^{35}S/^{3}H$ ratio.

served is one of conversion of short-lived precursors that lie far away from the zone we have analyzed in the two-dimensional electropherogram. In the experiment of Fig. 3*C*, a long-lived precursor that was converted to P during the chase period would have caused a rise in the curve. This was not seen. Thus, a long-lived precursor to P is not plausible.

Are P and Q microtubular proteins? These proteins can coassemble with porcine microtubular proteins when conditions are appropriate for polymerization. This serves as a strong operational criterion for microtubular proteins. Although the physical properties of P and Q resemble those of lower eukaryotic tubulins, further chemical characterization will be necessary before the identity of P and Q can be stated more definitely.

There are two extreme interpretations of the significance of the strong periodicity in synthesis of the *Physarum* microtubular proteins. One is that the periodicity stems from a homeostatic regulatory mechanism that maintains the level of the free microtubular protein pool as monomers are sequestered into microtubules. The other is that microtubular protein synthesis is directly involved in the causal chain of events leading to mitosis.

The interpretation that microtubular protein synthesis responds to microtubule formation is conceivable in *Physarum* because the total pool of these proteins in *Physarum* is relatively small [$\approx$0.2% (unpublished data)]. By contrast, in the sea urchin zygote, tubulin is 5% of total protein, and only 0.11% is needed to construct the mitotic spindle (17). An autoregulatory homeostatic loop controlling tubulin synthesis has been suggested by Ben-Ze'ev *et al.* (18) for HeLa and by Weeks and Collis (19) for flagellum formation in *Chlamydomonas reinhardtii*. This interpretation for the periodicity we observe faces the difficulty that synthesis increases long before metaphase signals the assembly of spindle microtubules.

In the alternative view, the proteins would play a causative role in regulating the mitotic cycle. When plasmodia on different mitotic schedules are fused, the heterokaryon goes through a synchronous mitosis at an averaged time (20). One explanation for this result is that a mitogen accumulates and then triggers mitosis when it reaches a critical level (see ref. 3 for a discussion). If the primary mitogen were to accumulate with linear kinetics, it might drive a change in the rate of synthesis of P and Q. This would result in an accelerative accumulation of P and Q. The spindle would then form with precipitous synchrony.

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