CELL CYCLE-DEPENDENT CHANGE IN THE PHOSPHORYLATION OF THE NUCLEOLAR PROTEINS OF *PHYSARUM POLYCEPHALUM* IN VIVO

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1. Introduction

Protein phosphorylating activity was shown to exist in the nucleoli of various cells such as rat liver [1,2], Novikoff hepatoma [3,4] and *Physarum polycephalum* [5,6]. The phosphorylating activity of nucleoli from Novikoff hepatoma cells is appreciably higher than that of normal liver nucleoli [7]. A nucleolar phosphoprotein subunit of 70 000 M_r has been isolated from nuclei of *Physarum polycephalum* after treatment of the nuclei with polyamines [8]. This phosphoprotein stimulated RNA polymerase I activity by 5-fold [8]. Thus, nucleolar protein phosphorylation might be concerned with the synthesis of ribosomal RNA precursors and their assembly into preribosomal particles required for the cell growth and division.

We have shown that in vitro phosphorylating activity of nucleoli of *Physarum polycephalum* increases 5-fold, reaching a maximum at 1.5 h before mitosis (late G2 phase) during the cell cycle [6]. This period of the maximal phosphorylating activity of the *Physarum* nucleoli in vitro coincides with that of morphological swelling [9,10] and ribosomal RNA synthesis of *Physarum* nucleoli [11–14]. Here, we examined whether the phosphorylation of *Physarum* is in fact increased before mitosis in vivo.

2. Materials and methods

Microplasmodia of *Physarum* were grown in shaken culture [15] and fused on filter papers as in [16]. After fusion at 25°C for 1.5 h, the growth medium, in which potassium phosphate was decreased by 80%, was poured into a petri-dish as in [17], and 2–20 μCi [^{32}P]orthophosphate and 0.05 ml hemin solution (2 mg/ml of 0.1% NaOH) were added to 20 ml media. Under these conditions, the second mitosis (M2) occurred at 17 h after the addition of growth medium and the third (M3) at 26 h. The stages of the cell cycle were ascertained by observation of wet-mounts under a phase contrast microscope. At various times after M2 and until M3, nucleoli were isolated from the macroplasmodia as in [18]. The contamination by nuclei was <5% of nucleoli as counted by hemocytometer. The continuously phosphorylated nucleoli (200 μg protein) were suspended in 5% trichloroacetic acid and heated at 90°C for 15 min to hydrolyze nucleic acids. To this solution, 0.1 ml 10% trichloroacetic acid and 0.1 ml bovine serum albumin (6.25 mg/ml) were added and cooled to 0°C. The radioactivity of the acid-insoluble material dissolved in 98% formic acid was measured in an Aloka-671 liquid scintillation counter. Proteins were determined as in [19]. Nucleoli were treated with 20 mM EDTA, dissolved in 0.1% sodium dodecyl sulfate (SDS) containing 62.5 mM Tris–HCl (pH 6.8) and 1% 2-mercaptoethanol, and boiled for 3 min. Polyacrylamide gel electrophoresis was performed as in [20]. The gel was stained with Coomassie blue. For autoradiography, the destained gel was dried, placed on an X-ray film (Kodak, OMAT R) and exposed for 10–14 days.

Nucleoli suspended in 5% trichloroacetic acid, were heated at 90°C for 15 min to hydrolyze nucleic acids and cooled to 0°C. The precipitated proteins were washed twice with cold 5% trichloroacetic acid. Then the alkali-labile phosphates were released from the acid-insoluble proteins by treatment with 1 N NaOH at 100°C for 5 min and measured by the ascorbic acid method [21].

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3. Results

Fig.1A shows the continuous incorporation of radioactive phosphate into nucleolar proteins of synchronous macroplasmodia at various times in the cell cycle. The specific activity (cpm/mg protein) decreased after M2, dropped to the basal level at 2 h after M2, then increased markedly in the G2 phase and reached a maximum at 6.25 h after M2, which is ~2 h before M3 (late G2 phase). The level of the maximal specific activity was 4-fold greater than the basal level. The specific activity, shortly after reaching the maximal level, dropped to the basal level again. Four such experiments were repeated with essentially the same results. This pattern of the change in specific activity of the nucleolar proteins observed in vivo is very similar to that observed for the phosphorylating activity of isolated nucleoli of *Physarum polycephalum* in vitro [6]. Fig.1B shows the incorporation of ^{32}P into nuclear proteins in vivo by the continuous labelling method similar to that conducted for nucleolar proteins. Nuclei were isolated according to [18]. There is no peak in the specific activity of nuclear proteins at ~1–2 h before M3 (fig.1B). Therefore, we can eliminate the possibility that the pattern in the change of specific activity of incorporated ^{32}P observed for nucleoli may be due to the incorporation of ^{32}P into contaminating nuclear proteins.

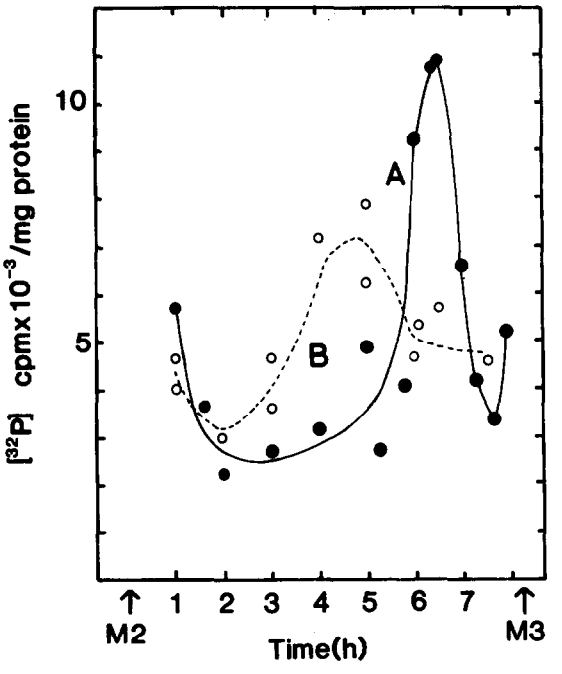

Fig.1. Continuous in vivo incorporation of ^{32}P from [^{32}P]-orthophosphate into nucleolar proteins of *Physarum polycephalum* at various times in the cell cycle. The ordinate shows cpm ^{32}P/mg protein. Macroplasmodia of *Physarum* were incubated with growth medium containing 2 μCi [^{32}P]-orthophosphate. The nucleoli were isolated from macroplasmodia at the times indicated as described in the text (A). For comparison, the cpm ^{32}P/mg nuclear protein were measured by the same procedure (B).

Nucleolar proteins continuously phosphorylated in vivo were analyzed by SDS gel electrophoresis and autoradiography. The electrophoretic patterns of nucleolar proteins which were stained, were almost the same throughout the cell cycle (fig.2A–E). The ^{32}P content of individual nucleolar proteins follows quite closely the cell cycle-dependent changes in the specific activity of nucleolar proteins (cf. fig.1A with fig.2A′–E′). During the first 6 h after M2 (fig.2A′–E′), nucleolar proteins of 125 000, 115 000 and 98 000 M_r were heavily phosphorylated. At 6.25 h after M2 (fig.2D′), these proteins were phosphorylated more heavily. In addition, nucleolar proteins of 67 000, 55 000, 34 000 and 27 000 M_r were significantly phosphorylated at this time in the cell cycle. These

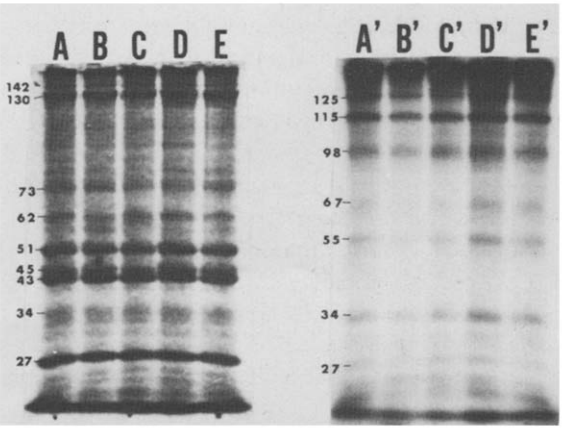

Fig.2. Electrophoretic profiles and autoradiographs of the nucleolar proteins phosphorylated in vivo during the cell cycle of *Physarum*, incubated continuously in the growth media containing 20 μCi [^{32}P]orthophosphate. The phosphorylated nucleoli (200 μg protein) were treated with 20 mM EDTA, solubilized with 0.1% SDS, and analyzed by SDS–polyacrylamide gel electrophoresis containing 10% acrylamide as in [20], and stained with Coomassie blue (A–E). The gel was dried, and then autoradiographed (A′–E′). Numerical values represent $M_r \times 10^{-3}$: (A,A′) 2.0 h after M2 (S phase); (B,B′) 5.25 h after M2 (middle G2 phase); (C,C′) 5.75 h after M2 (middle G2 phase); (D,D′) 6.25 h after M2 (late G2 phase); (E,E′) 7.0 h after M2 (late G2 phase).

nucleolar proteins phosphorylated in vivo are different from those phosphorylated in vitro [6] as judged from their M_r-values on SDS gel electrophoresis. Many nuclear proteins of *Physarum* are phosphorylated in vivo but not phosphorylated in vitro [22]. However, the nucleolar proteins of rat liver and hepatoma cells phosphorylated in vivo were the same as those phosphorylated in vitro [3,4,23,24]. In *Physarum*, probably the conditions for the phosphorylation of nucleoli in vitro cannot approximate the physiological circumstances.

4. Discussion

These results clearly indicate that both the specific activity and ^{32}P content of some nucleolar proteins of *Physarum* were increased markedly in vivo at 2 h before mitosis. Considering the large increase in in vitro phosphorylating activity of nucleoli isolated from *Physarum* at ~1.5–2.0 h before mitosis [6], the data indicate that a substantial increase in the phosphorylating activity of *Physarum* nucleoli must occur in situ in this period. This conclusion was further supported by the fact that the alkali-labile phosphate content of nucleolar proteins was 0.4 μmol/mg protein at 2 h before mitosis, which is significantly greater than the value (0.08–0.11 μmol) at other times in the cell cycle. Since this period of maximal specific activity in vivo, of maximal incorporation of ^{32}P in vitro and of maximal phosphate content coincides with that of morphological swelling [9,10] and ribosomal RNA synthesis of *Physarum* nucleoli [11–14], further work is needed to elucidate whether the marked phosphorylation of nucleolar proteins of *Physarum* just before mitosis may be related to the morphological change of the nucleoli or to the transcription of ribosomal DNA.

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