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

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1. Introduction

It has become a popular concept that protein phosphorylation may play an important role in the regulation of various cellular functions [1]. Recently, selective phosphorylation of nucleolar proteins of rat liver [2] and hepatoma ascites cells [3–5] was shown to occur both in vivo and in vitro. In these studies it was demonstrated that the proteins phosphorylated in vivo are phosphorylated in vitro as well. However, the physiological role of the phosphorylation of nucleolar proteins has not been elucidated yet.

Here we used nucleoli from *Physarum polycephalum. Physarum* is suitable for studying of the control mechanism involved in the biochemical events occurring in the cell cycle because the nuclei of macroplasmodia of *Physarum* undergo synchronous mitosis every 9–10 h. Here, we report that the endogenous phosphorylation of nucleolar proteins of *Physarum* increases remarkably in late G2 phase during the cell cycle.

2. Materials and methods

Physarum polycephalum microplasmodia (kindly supplied by Dr S. Matsumoto of the National Institute of Radiological Sciences) were grown in shake culture as in [6]. Fusions of macroplasmodia were conducted for 1.5 h on filter paper (15 cm diam.) supported by a layer of 8 mm glass beads in a petri dish at 25°C [7]. Under these conditions, the first mitosis occurred at ~6–7 h after the addition of growing medium to the fused macroplasmodia. The second mitosis (M2) was observed after 16 h and the third (M3) after 26 h. Nucleoli were isolated at various times after M2 and until M3. The stages of the cell cycle were ascertained by observation of wet-mounts under phase-contrast microscopy.

Nucleoli were isolated from the homogenates of macroplasmodia as well as microplasmodia by a modification of the method [8] in which $MgCl_2$ was substituted for $CaCl_2$. All procedures were done at 0–4°C. The contamination of nuclei was <5% as counted by hemocytometer.

The phosphorylation of nucleoli was carried out in the reaction mixture in 200 μl containing 150 μg nucleolar proteins, 50 mM Tris–HCl (pH 7.5), 0.1 M NaCl, 10 mM $MgCl_2$ and 5 μM [γ-^{32}P]ATP (100–200 cpm/pmol). After incubation for 15 min at 25°C, the reaction was stopped by adding 200 μl of 10% trichloroacetic acid. After hydrolysis of nucleic acids at 90°C for 15 min, the acid-insoluble material was collected by centrifugation and washed with cooled 5% trichloroacetic acid as in [9]. The radioactivity was measured in a Aloka-671 liquid scintillation counter with an efficiency of >80%.

Polyacrylamide slab gel electrophoresis was performed as in [10] using 10% acrylamide and 0.1% SDS. Gel was stained with Coomassie blue. For autoradiography, the destained gel was dried, placed on an X-ray film (Kodak, O-MAT R), and exposed for 10–14 days.

3. Results

When the nucleoli isolated from microplasmodia of *Physarum* were incubated with [γ-^{32}P]ATP in vitro as in section 2, a considerable incorporation of radioactivity into nucleolar proteins occurred. The incorporation of radioactive phosphate reached a maximum

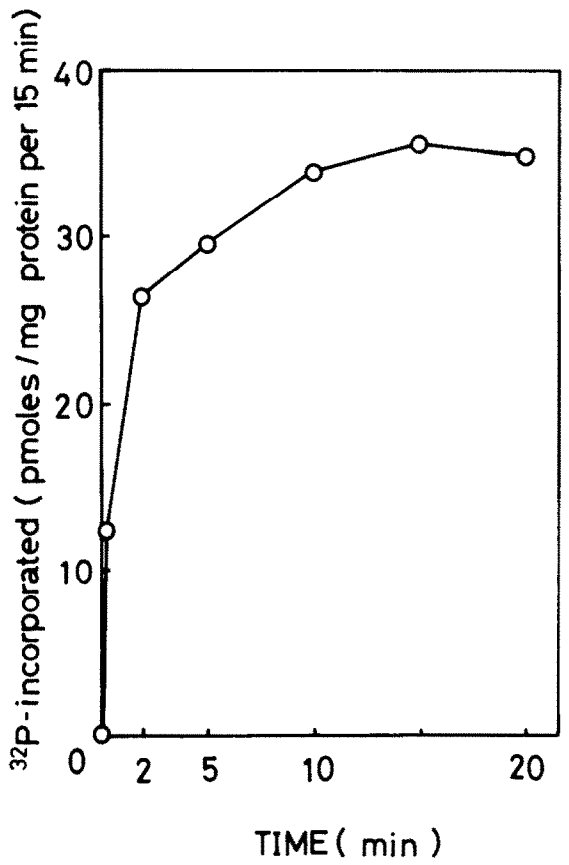

Fig.1. Time course of endogenous phosphorylation of nucleoli isolated from microplasmodia of *Physarum*. The nucleoli were incubated with [γ-^{32}P]ATP as described in the text, and 0.2 ml samples were periodically withdrawn at the times indicated.

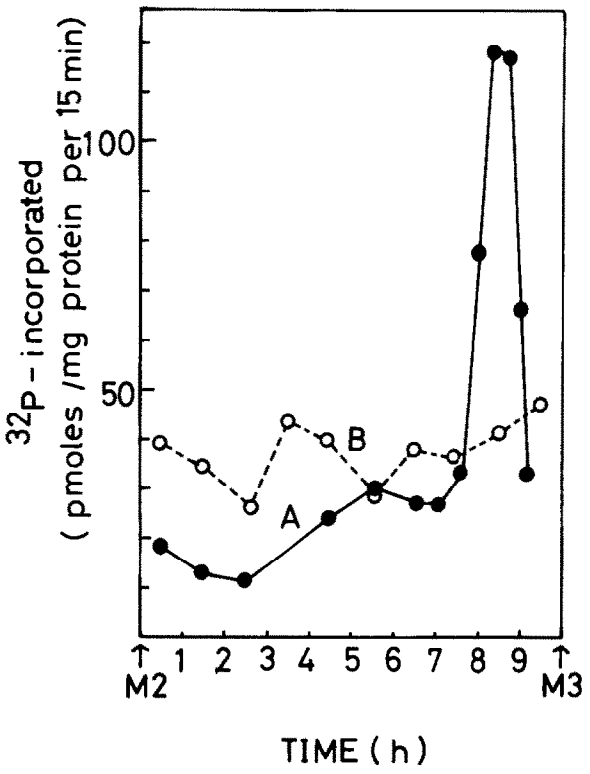

Fig.2. Changes in rate of endogenous phosphorylation of nucleoli (A) and nuclei (B) at different times in the cell cycle of *Physarum*. The nucleoli or nuclei were isolated from macroplasmodia at the times indicated in the cell cycle and were incubated with [γ-^{32}P]ATP under standard conditions as described in the text.

after 15 min incubation (fig.1). Thus 15 min incubation was chosen in the following studies. The phosphorylation reaction was sharply dependent on [Mg^{2+}] (not shown). The phosphorylation increased linearly as Mg^{2+} was increased to 10 mM. We used 10 mM $MgCl_2$ here because addition of >20 mM Mg^{2+} caused deformation of the nucleoli as judged by phase-contrast microscopy. Cyclic nucleotides, namely cyclic AMP and cyclic GMP had no appreciable effect on the phosphorylation over 10^{-8}–10^{-4} M. Therefore cyclic nucleotides were not added here.

Fig.2 shows the endogenous phosphorylation activity of nucleoli isolated from macroplasmodia of *Physarum* at various times in the cell cycle. The activity was almost unchanging for 7.5 h after M2, but increased markedly at 8.5 h after M2. The level of maximum activity was 4–5-fold greater than the basal level (fig.2A). Just before M3, the activity dropped to the basal level. This contrasts with phosphorylation of nuclei of *Physarum*. When isolated nuclei of *Physarum* were incubated with [γ-^{32}P]ATP under the same condition as above, the level of uptake of radioactive phosphate remained essentially constant throughout the cell cycle (fig.2B). This excludes the possibility that the phosphorylating activity change observed for nucleoli is due to the activity change of contaminating nuclei or chromatin-associated nuclear protein kinase.

To determine which of the nucleolar proteins are specifically phosphorylated, the nucleoli isolated at the different times in the cell cycle were phosphorylated, and then subjected to SDS gel electrophoresis and autoradiography. There were essentially no discernible differences in the electrophoretic patterns of nucleoli and in the autoradiograms of the phosphoryl-

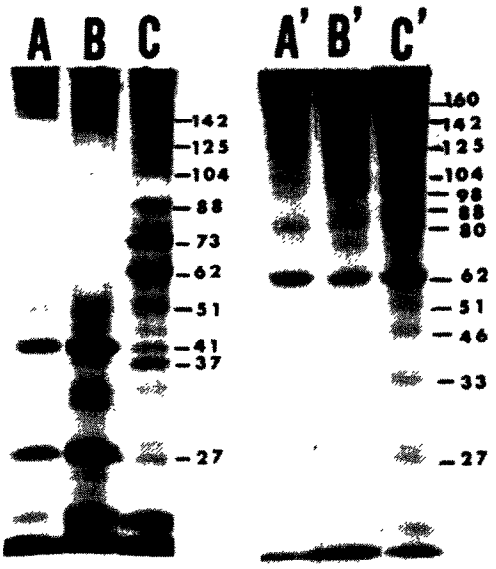

Fig.3. Electrophoretic profiles and autoradiographs of the nucleolar proteins isolated at different times in the cell cycle of *Physarum* and phosphorylated as in fig.2. The phosphorylated nucleoli (200 μg as protein) were solubilized with SDS and analyzed by SDS–polyacrylamide gel electrophoresis as in [10] and stained with Coomassie blue (A–C). The gel was dried, and then autoradiographed (A′–C′). Numerical values represent $M_r \times 10^{-3}$ (A,A′) 1.5 h after M2 (S phase); (B,B′) 5.5 h after M2 (early G2 phase); (C,C′) 8.5 h after M2 (late G2 phase).

Fig.4. Autoradiographs of the nucleolar proteins isolated at different times in late G2 phase. The experimental conditions were the same in fig.3. The nucleoli were isolated at 7.6 h (lane A), 7.8 h (lane B), 8.1 h (lane C), 8.4 h (lane D), 9.1 h (lane E), 9.5 h (lane F) after M2.

ated nucleolar proteins isolated at 1.5 h and 5.5 h after M2 (fig.3A,A′; B,B′). It is to be noted that quantitatively minor components of nucleolar proteins phosphorylated most strongly. In the nucleoli at 8.5 h after M2, 4 proteins with M_r 88 000, 73 000, 62 000 and 37 000 became more intensely stained (fig.3C), and proteins with M_r 41 000 and 27 000 were less intensely stained. The incorporation of radioactive phosphate into nucleolar proteins with M_r 160 000, 142 000, 125 000, 80 000, 62 000, 51 000, 46 000, 33 000 and 27 000 were greatly increased at 8.5 h after M2 (fig.3C′).

We examined the late G2 specific increase in the phosphorylation of nucleolar proteins in more detail (fig.4). The period in which the maximal incorporation of radioactive phosphate occurred was very short. The maximal incorporation was observed at 1.5 h before M3 (fig.4D) and the incorporation was abruptly decreased at 0.5 h before M3 (fig.4F).

4. Discussion

What is the physiological role of the marked phosphorylation of nucleolar proteins just before the mitosis? Histone kinase activities of nuclei of *Physarum* increased during the G2 phase and it was proposed that the phosphorylation of histone H1 is the initiation step for mitosis [11]. However, as judged from the reported M_r-values of histones of *Physarum*, 55 000, 40 000 and below 20 000 [12], most of the radioactive bands on the SDS gel here (fig.3,4) were not histones. Another interesting possibility is that RNA polymerase I might be phosphorylated in G2 phase, resulting in the activation of the enzyme. The M_r-values of RNA polymerase I in [13] of *Physarum* were 200 000, 135 000, 85 000, 45 000, 24 000 and 17 000. Since there are no phosphorylated bands corresponding to RNA polymerase I, this possibility seems less likely. The other candidates for the phosphorylated proteins

are preribosomal proteins and acidic proteins. A preliminary experiment indicated that when the phosphorylated nucleolar proteins were fractionated on their solubility [12], most of the phosphorylated proteins were extracted in phenol, suggesting that the proteins are acidic. Since acidic nucleolar proteins are thought to be involved in control of gene activity, the relation of the phosphorylated proteins to the transcription of ribosomal DNA deserves of further study.

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