Impact of nuclear actin on the prophase chromosome construction of *Physarum polycephalum* *

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Abstract A cell-free system efficiently promoting mitosis has been developed using the precise natural synchronous plasmodium of *Physarum polycephalum*. The content changes of nuclear cyclin B were exploited to represent the prophase process of *Physarum polycephalum*. The possible function of nuclear actin on chromosome construction was investigated by detecting the content changes of nuclear cyclin B in the late G_2 phase nuclei treated with cytochalasin B and incubated in the cell-free system. Our results showed that nuclear actin plays an important role in the process of the chromosome construction.

Keywords: *Physarum polycephalum*, nuclear actin, chromosome construction, cyclin B, cell-free system.

Actin is a highly conserved and abundant cytoskeletal protein in eukaryotic cells, and is proved to be essential for a number of cellular activities, including reorganization of cell shape and cell motility[1]. Recently, a huge amount of biochemical analyses and immunocytochemical experiments have proved that actin is present in nucleus[2~5], and several functions may be linked to nuclear actin, such as chromosome condensation[6], RNA transcription[7,8] and RNA transport[9]. In this article, we report the role of nuclear actin in the process of chromosome construction. In order to avoid the possible contamination of the cytosolic actin, we developed a cell-free system with the metaphase extracts of *Physarum polycephalum*, which can efficiently promote mitosis. Cyclin B is a key regulatory protein of the cell cycle and controls the G_2/M transition, and it is associated with the cdc2 kinase in G_2 phase to form a complex, which is involved in regulating the events of mitosis and is necessary for the movement from G_2 through M[10]. The content changes of nuclear cyclin B can represent the prophase process. Our results on the content changes of nuclear cyclin B in the late G_2 phase nuclei incubated in the cell-free system demonstrated that nuclear actin is involved in the prophase chromosome construction.

1 Materials and methods

1.1 Strain resource and culture methods

Strain TU_{291} of *Physarum polycephalum* was a gift from Dr. Philipe Albert, Cytobiology Laboratory of Reims University, France, and the culture method was from Daniel and Baldwin[11].

1.2 Isolation of *Physarum* nuclei

The isolation of nuclei of the late G_2 phase *Physarum* plasmodia was performed according to the procedure of Nothacher[12] and Mohberg[13] with appropriate modifications. Natural synchronous plasmodium of *Physarum polycephalum* was suspended in the precooled nuclear isolation buffer A containing 0.25 mol/L sucrose, 0.01 mol/L $MgCl_2$, 0.5 mmol/L PMSF, 0.01 mol/L Tris-HCl (pH 7.2), 0.6% surfynol, 1 mmol/L EGTA, then homogenized 50 times with a Dounce homogenizer. The homogenated solution was centrifugated at 2200 g for 4 min at 4 ℃. The pellet was resuspended in the nuclear isolation buffer B (1 mol/L sucrose, 0.01 mol/L $MgCl_2$, 0.5 mmol/L PMSF, 0.01 mol/L Tris-HCl (pH 7.2)) and centrifugated at 2500 g for 10 min at 4 ℃. The pellet was resuspended in the nuclear isolation buffer B with 30% Percoll and centrifugated at 2500 g for 10 min at 4 ℃. Isolated nuclei were washed twice with RSB (10 mmol/L NaCl, 5 mmol/L $MgCl_2$,

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10 mmol/L Tris-HCl (pH 7.4), 0.5 mmol/L PMSF), and pelleted by centrifugating for 10 min at 3000 g.

1.3 Mitotic extract preparation of *Physarum polycephalum*

Metaphase *Physarum* plasmodia were suspended in a mitotic extract buffer precooled in ice-water, containing 50 mmol/L Tris-HCl (pH7.3), 50 mmol/L KCl, 10 mmol/L $MgCl_2$, 20 mmol/L β-glycerophosphate, 10 mmol/L EGTA, 1 mmol/L DTT, 0.1 mmol/L PMSF and 10 μg/mL aprotinin, then homogenized 50 times with the Dounce homogenizer and centrifugated 5 min at 1000 g. The supernatant was collected and clarified by centrifugating at 50000 g for 5 min. The soluble mitotic extract was divided into 80 μL aliquots and stored under liquid nitrogen where it remained active for at least 2 months.

1.4 SDS-PAGE analyses

Specimens of nuclei and plasmodium were dissolved in Laemmli buffer[14], heated at 100 ℃ for 5 min, centrifugated to remove sediments. Gel electrophoresis was carried out in the presence of SDS according to Laemmli using a 10% separation and 4% concentration gel. The gel was stained with Coomassie Brilliant Blue (R-250) and decolored in alcohol-acetate.

1.5 Western blotting

The proteins on the gel were eletrotransferred to nitrocellulose (NC) membrane at 200 V for 2 h in the transfer buffer containing 25 mmol/L Tris, 192 mmol/L glycine, 20% methanol. The NC film was incubated in TBST (TBS containing 0.05% Tween 20) solution with 1% skimmed milk powder at room temperature for 1 h, washed three times in TBST for 5 min each. The film was incubated with rabbit anti-cyclin B antibody at a 1∶300 dilution in TBST at 37 ℃ for 1 h. After washing three times in TBST, the film was incubated with HRP-conjugated goat anti-rabbit IgG antibody at a 1∶1000 dilution in TBST at 37 ℃ for 30 min. The antigen-antibody complex was visualized by incubation of the washed film with 0.5 mg/mL 3-amino-9-ethylcarbazole (AEC) in TBS containing 0.006% H_2O_2 for 1 ~ 2 min. Quantification of the intensity of the protein bands was performed using a UVIband V99 software (UVItec St. John's Innovation Centre, Cambridge, UK).

1.6 Cell-free system

The mitotic extracts of *Physarum polycephalum* were supplemented with ATP to a final concentration of 3 mmol/L. Of the 30 μL late G_2 phase nuclei suspension was incubated in the presence of 120 μL mitotic extracts at 26 ℃ for 0 min, 20 min, 40 min and 60 min, respectively, and the mitotic extract buffer was used as control. The nuclei were collected by centrifugating at 3000 g for 3 min. After washing 3 times with mitotic extract buffer, the nuclei were lysed in SDS sample buffer for SDS-PAGE and Western blot analysis.

1.7 Treatment of nuclei with cytochalasin B

The late G_2 phase nuclei were treated with 5 μmol/L cytochalasin B (dissolved by 2% DMSO), and then incubated in mitotic extracts at 26 ℃ for 0 min, 20 min, 40 min and 60 min, respectively. The nuclei treated with 2% DMSO was used as control. The treated nuclei were collected and lysed in SDS sample buffer for SDS-PAGE and Western blotting.

2 Results

2.1 Synchronous endonuclear mitosis of *Physarum polycephalum*

The life cycle of *Physarum polycephalum* undergoes amoebae stage, plasmodial stage and sporulation stage. The plasmodium of *Physarum polycephalum* is multinucleated and exhibits naturally synchronous mitosis. All nuclei within a single plasmodium divide synchronously, and the cell cycle is about 10 h at 26 ℃, which consists of S phase, G_2 phase and mitosis, and lacks G_1 phase. The S phase and the G_2 phase last 3 h and 6 h, respectively. The progress of the cell cycle of *Physarum* plasmodium was monitored by examining the morphology of nuclei under a light microscope every 5 ~ 10 min. The cell cycle of *Physarum polycephalum* is shown in Fig. 1.

2.2 The changes of cyclin B content in the cell cycle of *Physarum polycephalum*

To detect the cyclin B content changes in the cell cycle of *Physarum polycephalum*, the *Physarum* plasmodia at different phases were collected and the Western blot analysis using anti-cyclin B antibody was performed. The protein analysis of *Physarum* plasmodia showed that cyclin B was present in the plasmodia of S phase, G_2 phase, prophase and

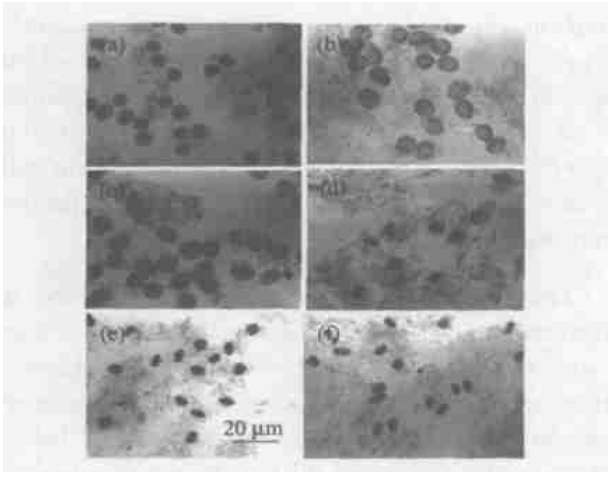

Fig. 1. Nuclei of synchronous plasmodia of *Physarum polycephalum*. (a) S phase nuclei, nucleolus and chromatin cannot be easily distinguished; (b) G_2 phase nuclei, nucleolus is large and situated in the center of the nuclei; (c) prophase nuclei, nucleoli migrates to the edge of the nuclei; (d) pro-metaphase nuclei, chromosome is dispersed in the nuclei; (e) metaphase nuclei, replicated chromosomes align at the center of the nuclei; (f) anaphase/telophase nuclei, chromosomes migrate to opposite poles of the nuclei. The plasmodia were stained with Carbol fuchsine.

metaphase. The molecular weight of Cyclin B in *Physarum polycephalum* was around 62kD. Cyclin B was gradually accumulated from S phase to metaphase, and reached the peak at metaphase, but disappeared in anaphase and telophase (Fig. 2). Cyclin B is a key regulatory protein of cell cycle and is essential to the control of the G2/M transition. So, the content changes of nuclear cyclin B may be used as a marker for the prophase process.

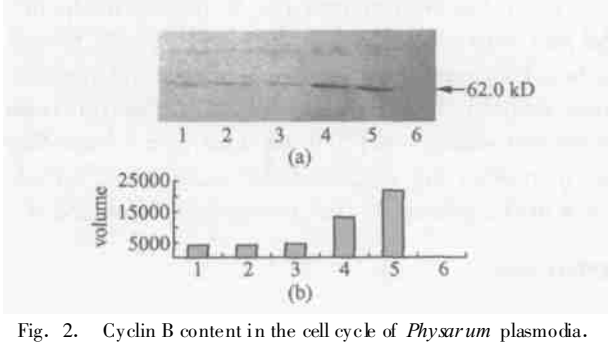

Fig. 2. Cyclin B content in the cell cycle of *Physarum* plasmodia. (a) Cyclin B detected by Western blotting, (b) analysis of protein quantification. Lanes 1 ~ 6 are specimens of S phase, early G_2 phase, late G_2 phase, prophase, metaphase, and anaphase/telophase, respectively.

2.3 The cell-free system of nuclear mitosis of *Physarum polycephalum*

Mitotic extracts were prepared from the synchronous metaphase plasmodia of *Physarum polycephalum*. The results of SDS-PAGE and Western blotting indicated that the mitotic extracts contained plenty of proteins and cyclin B was a component of the mitotic extracts of *Physarum polycephalum* (Fig. 3). When the late G_2 phase nuclei of *Physarum* were incubated in the cell-free system, we found that cyclin B in the nuclei gradually increased with the prolongation of incubating time. While, in the control group, the content of cyclin B almost did not change (Fig. 4(a)). This indicated that the mitotic extracts of metaphase plasmodia have the activity of accelerating the process of prophase, and the cell-free system with MPF activity was established.

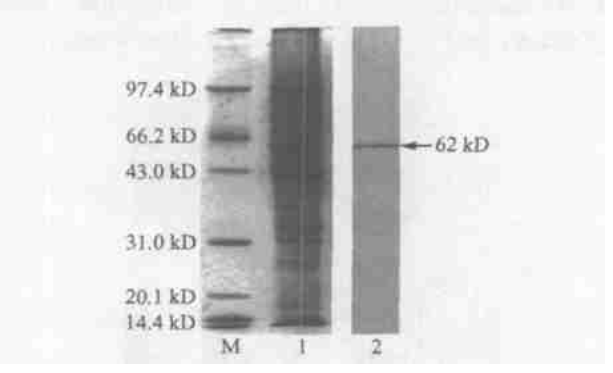

Fig. 3. SDS-PAGE and Western blot analysis of the mitotic extracts. M, Protein standard; 1, mitotic extracts; 2, cyclin B detected.

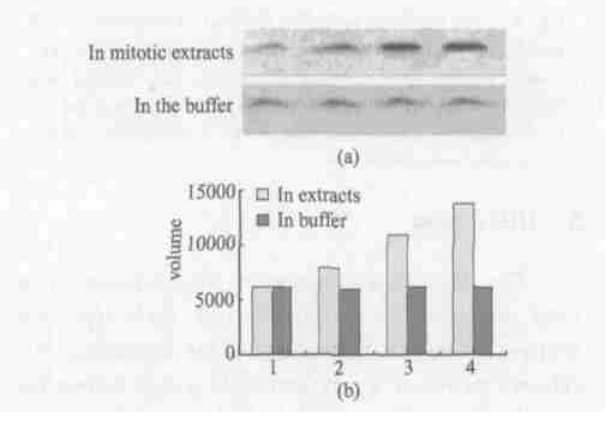

Fig. 4. The content changes of cyclin B in the late G_2 phase nuclei incubated in the mitotic extracts. (a) Western blot analysis using anti-cyclin B antibody, (b) protein quantification. 1 ~ 4 are the nuclei specimens incubated for 0 min, 20 min, 40 min and 60 min, respectively.

2.4 Impacts of nuclear actin on the prophase chromosome construction of *Physarum polycephalum*

Cytochalasin B is a functional inhibitor of actin that can combine with the N-terminal of actin and block the growth of actin filament. The late G_2 phase

nuclei treated by 5 μmol/L cytochalasin B were incubated in the mitotic extracts from the metaphase plasmodia. After carefully washing, Western blot analysis was conducted, and the results showed that the content of cyclin B in the nuclei changed little. In contrast, the content of cyclin B gradually increased with the prolongation of incubating time in the control group (Fig. 5(a)). Our results indicated that the treatment of nuclei with cytochalasin B affected the increase of the cyclin B content in the nuclei. As cyclin B content changes can represent the prophase process, we inferred that cytochalasin B destroyed the polymerization of the nuclear actin and directly affected chromosome construction of *Physarum polycephalum*, and arrested the process of prophase.

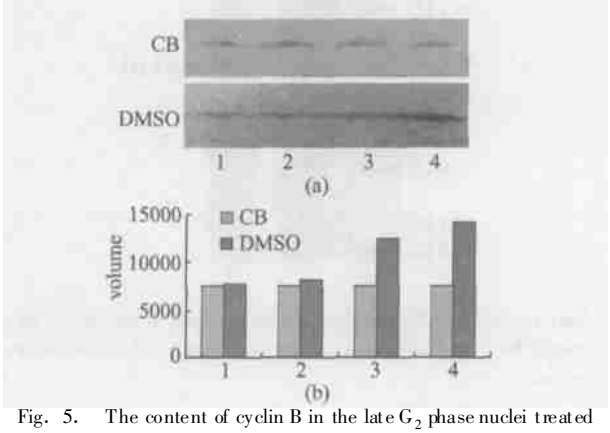

Fig. 5. The content of cyclin B in the late G_2 phase nuclei treated with CB or 2% DMSO incubated in the metaphase extracts. (a) Western blotting using anti-cyclin B antibody. CB, Treated with CB; DMSO, treated with 2% DMSO. (b) Protein quantification. 1 ~ 4 are the nuclei specimens incubated for 0 min, 20 min, 40 min and 60 min, respectively.

3 Discussion

The slime mold *Physarum polycephalum* is an ideal organism to investigate cell cycle-dependent changes in chromatin structure. This organism can be cultured as a giant syncytium with several million nuclei that are perfectly synchronized throughout the cell cycle. Thus, cellular extracts from macroplasmodia of *Physarum polycephalum* can be easily prepared from precise points during the cell cycle without the use of exogenous stage-specific blocking or synchronizing agents that may interfere with or alter the biochemical activities within the extracts. Hobohm et al. observed that the extracts of plasmodia homogenized 45 min before late telophase accelerated the onset of mitosis in the microinjected plasmodium up to 70 min[15]. Christophe et al. found that extracts harvested at the end of G_2 phase *Physarum polycephalum* caused increases in the nuclear volume of chicken erythrocytes[16]. Enlightened by all this work, we have developed a cell-free system from the precise natural synchronous plasmodium of *Physarum polycephalum*, which is efficiently promoting mitosis, to study the role of nuclear actin in the prophase chromosome construction.

The mechanism of chromatin condensation into chromosome is unclear. Chromosome construction is an important event in cell division and regulation. The role of actin in the prophase chromosome construction is largely unknown. Rungger et al. believed that actin was associated with chromosome condensation because the microinjection of anti-actin antibody into Amphibian oocyte could block the chromosome condensation[6]. Using the specific actin disrupting agent cytochalasin D, Sauman et al. have demonstrated the structural significance of nuclear actin in maintaining the linear integrity of polytene chromosomes[17]. Song et al. observed that mitosis and chromosome condensation in the CB-treated specimens began later than that of the control[18]. But they did not prove the role of nuclear actin in chromosome construction and cell cycle, because in that experiment cytochalasin B was microinjected into *Physarum* plasmodia. By detecting the content changes of nuclear cyclin B in the late G_2 phase nuclei incubated in the cell-free system, we showed that nuclear actin is involved in the prophase chromosome construction.

Actin has been indicated to be present in the nuclei and chromosome in eukaryotic cells, and proved to be a component of the nuclear matrix and chromosome scaffold. But the knowledge about the functions of nuclear actin is very limited until now. According to our results, we suggested that nulear actin participates in the process of the chromosome construction.

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