Function of c-Fos-like and c-Jun-like Proteins on Trichostatin A-induced G$_2$/M Arrest in *Physarum polycephalum*

Xiao-Xue LI, Jun LU, Yan-Mei ZHAO, and Bai-Qu HUANG*

Institute of Genetics and Cytology, Northeast Normal University, Changchun 130024, China

Abstract   The homologs of transcription factors c-Fos and c-Jun have been detected in slime mold *Physarum polycephalum* during progression of the synchronous cell cycle. Here we demonstrated that c-Fos-like and c-Jun-like proteins participated in G$_2$/M transition by the regulation of the level of Cyclin B1 protein in *P. polycephalum*. The study of antibody neutralization revealed that interruption of the functions of c-Fos-like and c-Jun-like proteins resulted in G$_2$/M transition arrest, implicating their functional roles in cell cycle control. When G$_2$/M transition was blocked by histone deacetylase inhibitor trichostatin A, changes in c-Fos- and c-Jun-like protein levels, and hyperacetylation of c-Jun-like protein, were observed. The data suggest that in *P. polycephalum*, c-Fos- and c-Jun-like proteins may be the key factors in the regulation of histone acetylation-related G$_2$/M transition, involving the coordinated expression and hyperacetylation of these proteins.

Key words   c-Fos-like protein; c-Jun-like protein; trichostatin A; G$_2$/M transition; *Physarum polycephalum*

The acetylation status of core histones plays an essential role in the transcriptional regulation of many genes, and it is also involved in the proliferation and differentiation of eukaryotic cells [1]. Histone acetylation and deacetylation are governed by the reverse action of two kinds of enzymes, the histone acetyltransferases (HATs) and the histone deacetylases (HDACs). Histone deacetylation may repress transcription by strengthening histone-DNA interaction and thereby blocking the access of transcriptional regulators to the DNA template [2–4]. Inhibition of HDACs can regulate the transcriptional activation of specific genes through the modulation of chromatin conformation that is caused by histone hyperacetylation. HDAC inhibitors (HDIs) have been reported to induce G$_1$ or G$_2$ phase arrest, and regulate the transcription of a number of cell cycle regulator genes, including *p21*, *c-myc*, *cyclin* and *cdk* [5–7]. Indeed, HDIs provide a useful and convenient means to investigate the relationship be-

tween histone acetylation and the regulation of cell cycle checkpoint transition.

In eukaryotes, the fundamental regulators that control cell cycle progression include Cyclins, Cyclin-dependent kinase (CDK) and CDK inhibitors. CDK/Cyclin complexes are the ultimate regulatory components that directly determine the transition of checkpoints in cell cycle progression [8,9]. It has been reported that in mammalian cells trichostatin A (TSA), one of the HDIs, blocked G$_2$/M transition, and downregulated the transcription and translation of Cyclin B1 [10]. An immediate point of attention is whether and how other cell cycle relating factors function in TSA-induced G$_2$/M transition.

Physarum polycephalum, a naturally synchronized slime mold, has a similar mechanism of cell cycle control to mammalian cells, and information about identification and functions of HATs and HDACs in *P. polycephalum* has been reported previously [11–14]. It has been demonstrated that TSA induced a significant delay in G$_2$/M transition [11]. c-Fos and c-Jun are components of transcriptional factor AP-1 (activating protein-1). They were shown to interact with the promoters of a number of cell cycle genes and play important roles in cell cycle regulation [15]. The

homologs of these two factors were found in *P. polycephalum* [16], but their functions have not been intensively studied.

In this study, the hyperacetylation of c-Jun-like protein and changes in c-Fos- and c-Jun-like protein levels were observed in TSA-induced G_2/M transition block in *P. polycephalum*. The results suggest that c-Fos- and c-Jun-like proteins, as well as Cyclin B1, may be functional molecules in histone acetylation-related G_2/M transition control in *P. polycephalum*.

Materials and Methods

Materials

Strain TU291 of *P. polycephalum* was a gift from Dr. Philipe ALBERT (Cytobiology Laboratory, Reims University, Reims, France). Suspension and synchronous cultured methods of *P. polycephalum* were adopted from Daniel and Baldwin [17]. The plasmodia were cultured on MSD medium at 26 ℃.

Antibody neutralization experiments

Equal segments of the synchronous G_2 plasmodia were starved for 1.5 h in the dark to absorb protein, then cultured on the medium with antibodies (antibody treatment) or without (control). The antibodies used were rabbit polyclonal antibodies against human whole c-Fos and c-Jun proteins (Zhongshan Biotechnology, Beijing, China). The anti-c-Fos and anti-c-Jun antibodies were dissolved in medium at 1:500 and 1:50 dilution, respectively, and the final concentration of each was 0.4 μg/ml. Cell cycle progression of the plasmodia was monitored and recorded by light microscopy.

Two hundred milligrams of both the plasmodia after antibody treatment and the control were disrupted by repeated Dounce homogenization in pre-cooled cell extraction buffer [0.25 M sucrose, 10 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.2, 0.2% (*W/V*) Triton X-100, 0.5 mM phenylmethylsulphonyl fluoride] at a ratio of 0.2 g wet weight per milliliter. Then they were sonicated at 4 ℃ before an equal volume of sample buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% sodium dodecyl sulphate, 0.2% bromophenol blue, 20% glycerol) was added. Twenty microliters of sample lysate was added to each lane for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gel.

For Western blot analysis, the separated proteins were transferred to nitrocellulose membranes after electrophoresis. The membranes were soaked for 1 h in blocking buffer (2% non-fat dry milk in phosphate-buffered saline, pH 7.4), then incubated at 37 ℃ for 1 h with rabbit polyclonal antibodies against human whole Cyclin B1 (a dilution of 1:1000 in the blocking buffer; Santa Cruz Biotechnology, Santa Cruz, USA) and human whole anti-actin antibodies (a dilution of 1:500 in the blocking buffer; Zhongshan Biotechnology). After incubation with horse-radish peroxidase (HRP)-conjugated secondary antibody (Zhongshan Biotechnology), the membranes were visualized by staining with 3-amino-9-ethylcarbazole.

The semi-quantitative estimation of the Cyclin B1 expression level was accomplished by analyzing the relative ratio between the photodensitometric values of Cyclin B1 and actin (the internal control) bands.

TSA treatment of plasmodia

TSA treatment was carried out in a similar manner to that of antibody treatment. The G_2 macroplasmodia were first starved for 1.5 h, then cultured on the medium with 10 μM TSA (TSA treatment) or without (control). The cell cycle of the plasmodia was monitored by light microscopy.

The proteins were extracted, separated by SDS-PAGE and transferred to nitrocellulose membranes, as described above. Anti-c-Fos and anti-c-Jun antibodies were used in Western blot at dilutions of 1:500 and 1:200, respectively. The semi-quantitative estimation of c-Fos- and c-Jun-like protein levels was accomplished by analyzing the relative ratio between the photodensitometric values as described above.

### Examination of acetylation level of c-Jun-like protein following TSA treatment

The proteins of G_2 plasmodia that underwent TSA treatment for 1 h and 3 h, and the control, were immunoblotted with anti-acetylated lysine (Cell Signaling Technology, Beverly, USA) and anti-c-Jun antibodies on the same membrane. After incubation with HRP-conjugated secondary antibody (Zhongshan Biotechnology), the membranes were visualized by staining with 3-amino-9-ethylcarbazole.

Results

Roles of c-Fos- and c-Jun-like proteins in cell cycle control

Before antibody neutralization experiments, the specificity between the antibodies (anti-c-Fos, anti-c-Jun and

anti-Cyclin B1) and the protein homologs in G_2 plasmodia were verified. All proteins in G_2 phase were separated by SDS-PAGE and subjected to Western blotting. The specific immunoblotting bands of c-Jun, c-Fos and Cyclin B1 were shown at around 39 kDa, 50 kDa and 62 kDa, respectively (**Fig. 1**). This suggested that the anti-c-Fos, anti-c-Jun and anti-Cyclin B1 antibodies reacted specifically with the homologs in G_2 plasmodia.
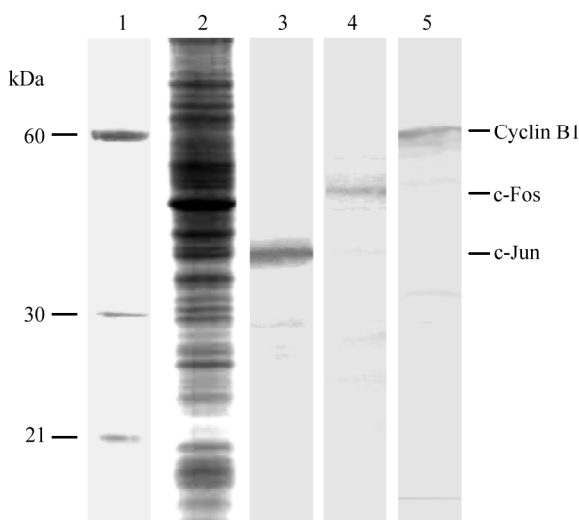
Fig. 1   Specific reactions between anti-c-Fos, anti-c-Jun and anti-Cyclin B1 antibodies and the corresponding protein homologs in G_2 plasmodia

G_2 plasmodia were disrupted by repeated Dounce homogenization in cell extract buffer and sonication. All proteins were subjected to 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Western blot analysis. 1, low molecular weight protein marker; 2, proteins of G_2 plasmodia; 3–5, the immunoblotting bands of c-Jun, c-Fos and Cyclin B1 at around 39 kDa, 50 kDa and 62 kDa, respectively.

Under the light microscope, it was observed that the G_2 plasmodia of *P. polycephalum* without antibody treatment (control) began mitosis normally [**Fig. 2(A)**], and progressed into metaphase after 4 h [**Fig. 2(B)**]. Meanwhile, the cell cycle of G_2 plasmodia with the treatment of anti-c-Fos or anti-c-Jun antibodies were both blocked in G_2 phase [**Fig. 2(C,D)**], unable to pass the G_2/M checkpoint. Furthermore, we treated the plasmodia with a mixture of the antibody and c-Fos- or c-Jun-like protein extracted from plasmodia, and found that the cell cycle progressed normally in the same manner as the control (data not shown). This experiment testified that the antibody reacted specifically with c-Fos- or c-Jun-like proteins. These results demonstrated that, as in mammalian cells, c-Fos- and c-Jun-like proteins played functional roles in G_2/M
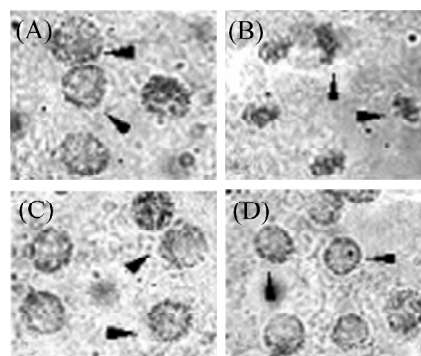

Fig. 2 G_2/M transition arrest in *Physarum polycephalum* with anti-c-Fos and anti-c-Jun antibody treatments

The cell cycle of *P. polycephalum* consists of S, G_2 and M phases, without G_1 phase. (A) View of the nuclei of G_2 plasmodia using light microscopy. (B) After 4 h, the G_2 nuclei without antibody treatment went into metaphase; but the G_2 nuclei with anti-c-Fos antibody treatment (C) or anti-c-Jun antibody treatment (D) were all arrested in G_2 phase. Magnification, 2000×.

checkpoint control in *P. polycephalum*. The interruption of the function of these two proteins by their antibodies resulted in arrest of G_2/M transition.

Fig. 3(A,B) shows the results of Western blot analysis of Cyclin B1 in G_2 plasmodia after anti-c-Fos and anti-c-Jun antibody neutralization. It can be seen from **Fig. 3(C)** that both anti-c-Fos and anti-c-Jun antibody treatments caused a decline in the Cyclin B1 level in G_2 phase. Cyclin B1 forms a complex with cdc2, which is essential for controlling the checkpoint of G_2/M in all eukaryotes [18, 19]. It has been reported that in *P. polycephalum*, Cyclin B1-like protein appeared in S phase, accumulated gradually from then on, peaked at metaphase and disappeared at telophase [20]. In this study, we demonstrated that loss of the functions of c-Fos- and c-Jun-like proteins resulted in the decrease of Cyclin B1-like protein in G_2 plasmodia. Insufficient Cyclin B1-like protein may be the direct reason for the block of G_2/M transition. These results suggest that the transcription factors c-Fos and c-Jun may participate in G_2/M transition by regulating Cyclin B1-like protein in *P. polycephalum*.

In endometrial cancer cells, some members of AP-1 were shown to interact with promoters of cell cycle genes, for instance, c-Fos interacted with promoters of *cyclin E* and *cyclin B1*, and Jun-B with that of *cyclin D1* [15]. c-Fos and c-Jun are two members of AP-1. Therefore, we presume that in *P. polycephalum* c-Fos- and c-Jun-like proteins may also interact with the promoters of cell cycle genes, such as *cyclin B1*. However, this presumption needs to be confirmed by further research.

(A)

(B)

(C)
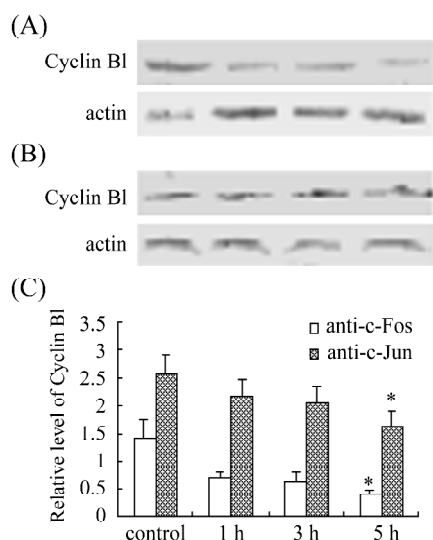
Fig. 3   Decline of Cyclin B1 levels in G$_2$ plasmodia after anti-c-Fos and anti-c-Jun antibody treatments
The G$_2$ plasmodia were treated with anti-c-Fos (A) or anti-c-Jun antibody (B) for 1 h, 3 h and 5 h. The Western blot bands of the internal reference (actin) and Cyclin B1 are presented on the top. The results of photodensitometric analysis are shown in (C). The experiment was repeated at least three times. The difference was significant (*$P<0.05$ vs. control). control, G$_2$ plasmodia without antibody treatment.

Block of G$_2$/M transition by TSA

The cell cycle progression of plasmodia was examined microscopically after 10 μM TSA treatment in G$_2$ phase. It was found that after 4 h the control G$_2$ plasmodia cultured in the medium without TSA [**Fig. 4(A)**] progressed normally into the next mitosis [**Fig. 4(B)**], but the TSA-treated plasmodia failed to progress into the next cycle of division, and were arrested in G$_2$ phase [**Fig. 4(C)**]. Thus, TSA was able to block the transition of G$_2$/M in *P. polycephalum*.

(A)   (B)   (C)
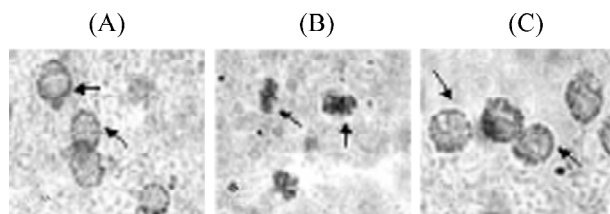
Fig. 4   Blockage of G$_2$/M transition in *Physarum polycephalum* induced by trichostatin A (TSA)
The G$_2$ nuclei (A) without TSA treatment underwent normal mitosis, which progressed into metaphase (B) after 4 h; but the G$_2$ nuclei were arrested at G$_2$ phase (C) by 10 μM TSA treatment. Magnification, 2000×.

TSA-induced changes in c-Fos- and c-Jun-like protein levels in G$_2$ phase

To examine whether changes in expression of c-Fos- and c-Jun-like proteins occur during TSA-mediated cell cycle arrest, Western blot analysis was performed after the plasmodia were treated with 10 μM TSA in G$_2$ phase. The results are shown in **Fig. 5(A)**. The photodensitometric data of the blots are presented in **Fig. 5(B)**. The results demonstrate that in G$_2$ phase, the c-Jun-like protein level was decreased, but c-Fos-like protein expression was increased with TSA treatment, which further suggested the regulatory roles played by these proteins in G$_2$/M transition.

(A)

(B)
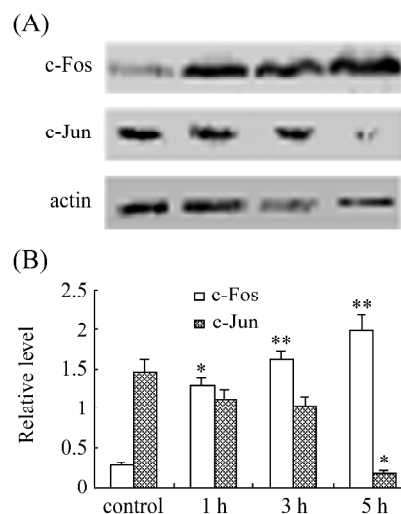
Fig. 5   Changes in c-Fos and c-Jun protein levels in G$_2$ plasmodia after trichostatin A (TSA) treatment
The G$_2$ plasmodia of *Physarum polycephalum* were cultured in medium containing 10 μM TSA for 1 h, 3 h and 5 h. The proteins were then subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Western blot analysis. The bands of internal reference (actin) and c-Fos and c-Jun proteins are indicated in (A). (B) shows the data of photodensitometric analysis of (A). The experiment was repeated at least three times. The differences were significant (*$P<0.05$ vs. control) and highly significant (**$P<0.01$ vs. control). control, G$_2$ plasmodia without TSA treatment.

Hyperacetylation of c-Jun-like protein following TSA treatment in G$_2$ phase

We then wanted to test whether the inhibition of histone deacetylase activity by TSA would influence the acetylation of these two transcription factors. Proteins of G$_2$ plasmodia treated with 10 μM TSA for 1 h or 3 h, and the control, were separated by gel electrophoresis and transferred to a nitrocellulose membrane. After immunoblotting

with anti-acetylated lysine and anti-c-Jun antibodies in sequence, the positive bands of acetylated lysine appeared at the position of c-Jun-like protein (**Fig. 6**), indicating that c-Jun-like protein was acetylated following TSA treatment. It is also clearly shown in **Fig. 6** that the acetylated c-Jun-like protein level in G_2 plasmodia with TSA treatment for 1 h or 3 h was higher than that in the control (**Fig. 6**). This indicates that in G_2 plasmodia, the acetylation degree of c-Jun-like protein increased after TSA treatment. The acetylation level of c-Fos-like protein did not undergo a significant change after TSA treatment (data not shown).
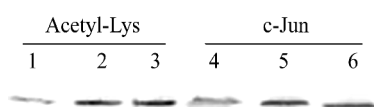
Fig. 6   Hyperacetylation of c-Jun-like protein in G_2 plasmodia induced by trichostatin A (TSA)

The proteins extracted from G_2 plasmodia were separated by gel electrophoresis and immunoblotted with anti-acetylated-lysine antibody (lanes 1–3) and anti-c-Jun antibody (lanes 4–6) on one single membrane. 1 and 4, without TSA treatment; 2 and 5, 10 μM TSA treatment for 1 h; 3 and 6, 10 μM TSA treatment for 3 h.

Discussion

TSA inhibits the activity of histone deacetylases at low nanomolar concentrations and causes cell cycle arrest at G_1/S or G_2/M in mammalian cells. It specifically upregulates or downregulates genes related with cell proliferation, apoptosis and differentiation, such as *cyclin B1* and *p21*, but does not affect housekeeping genes [10, 21]. In this study, we found that TSA blocked G_2/M transition in *P. polycephalum* (**Fig. 4**), and stimulated c-Fos-like protein expression (**Fig. 5**). The c-Jun-like protein level was decreased (**Fig. 5**), but it was hyperacetylated by TSA treatment (**Fig. 6**). These results suggest that c-Fos- and c-Jun-like proteins may participate in TSA-induced G_2/M transition arrest by regulating both their acetylation status and expression levels.

It was reported that some transcription factors recruited and reacted with HATs and HDACs to regulate the activities of cell cycle-related genes [22]. In G_1/S transition of mammalian cells, HDAC1 was recruited to transcriptional factor E2F by retinoblastoma protein Rb, and repressed the E2F-regulated promoter of the gene encoding the cell cycle regulator Cyclin E [22]. A histone deacetylase PpHDAC1, the homolog of RPD3 [23], was identified in

P. polycephalum [11]. Whether c-Fos- and c-Jun-like proteins can recruit PpHDAC1 or other HDACs to regulate the cell cycle-related genes is worthy of further study.

There has been evidence that certain histone acetyltransferases, such as P/CAF and p300/CBP, are able to acetylate some non-histone proteins, including the activators p53, Tat, GATA-1, and some transcriptional factors TFIIE and TFIIF [24,25]. In *P. polycephalum*, one cytoplasmic B-type and four nuclear A-type HATs have been identified [14]. However, acetylation of non-histone proteins in *P. polycephalum* has not been reported. We found that c-Jun-like protein was hyperacetylated by TSA treatment, but elucidation of the mechanism of this action will require more in-depth investigation.

To summarize, data presented in this study testified, for the first time, that c-Fos- and c-Jun-like proteins functioned in the regulation of G_2/M transition. The results also showed that the changes in expression levels of c-Fos- and c-Jun-like proteins, as well as the acetylation level of c-Jun-like protein, were related to the TSA-induced G_2/M arrest in *P. polycephalum*. These data suggested that c-Fos- and c-Jun-like proteins were two key factors in cell cycle checkpoint control in *P. polycephalum*.

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