ARTICLE

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A PSTAIRE CDK-like protein localizes in nuclei and cytoplasm of *Physarum polycephalum* and functions in the mitosis

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ABSTRACT

CDKs play key roles in controlling cell cycle progression in all eukaryotes. In plants, multiple CDKs are present, among which the best characterized CDKs are PSTAIRE CDKs. In this study, we carried out Western blot, immunoelectron microscopy and antibody treatment with an anti-PSTAIRE monoclonal antibody to explore the subcellular localization and functions of PSTAIRE CDKs in *Physarum polycephalum*. The results of Western blot and immunoelectron microscopy showed that in *P. polycephalum*, a PSTAIRE CDK-like protein was 34 kD in molecular weight and located in both nuclei and cytoplasm. In nuclei, the protein was mainly associated with chromosomes and nucleoli. The expression of the PSTAIRE CDK-like protein in both the plasmodia and nuclei showed little fluctuation through the whole cell cycle. When treated with an anti-PSTAIRE monoclonal antibody at early S phase, the cells were arrested in S phase, and the mitotic onset of *P. polycephalum* was blocked for about 1 h when treated at early G2 phase. Our data indicated that the PSTAIRE CDK- like protein has a direct bearing on the mitosis.

Keywords: PSTAIRE CDK-like protein, *Physarum polycephalum*, mitosis, immunoelectron microscopy.

INTRODUCTION

The cell cycle in eukaryotic organisms is primarily controlled by cyclin-dependent kinase (CDKs) in complexes with their activating and substrate-specifying partners, cyclins[1]. Based on sequence similarity, plant CDKs can be subdivided into five classes: CDKs A, B, C, D and E [2]. CDK A is the most numerous class. This class comprises CDKs that are most closely related to the prototypical CDKs (i.e., yeast cdc2/CDC28, animal cdc2/CDK1 and CDK2) and contains an evolutionary conserved 16 amino acid sequence called PSTAIRE (EGVPSTAIREISLLKE) motif, which is essential for cyclin binding [1, 2, 5, 6]. CDK B makes up a class of plant-specific CDKs which bear their unique motif, either PPTALRE (B1 group) or PPTTLRE (B2 group) and may be involved in the control of the G2/M progression in plants[3]. CDK C (with PITAIRE motif) [2], CDK D (with NFTALRE motif) [2, 4] and CDK E (with SPTAIRE motif)[2] form less numerous classes. Currently, CDK A and B are well defined, yet other classes are represented only by one or two known members whose distribution in the plant kingdom remains unclear. The best characterized plant CDK belongs to CDK A or PSTAIRE CDKs. PSTAIRE CDKs can partially rescue yeast cdc2/CDC28 mutations and are therefore supposed to be functional homologs of the yeast CDKs[3], and play important roles in cell cycle control[1, 5-10]. PSTAIRE CDKs are regulated at several levels, including its expression, differential subcellular localization, phosphorylation, protolysis, and interaction with regulatory proteins. Investigations upon the subcellular localization[11-13] and functions[7-10] of PSTAIRE CDKs have been limited, and opinions on their subcellular location have been divergent [11-13].

Physarum polycephalum is suitable to be used to study the biochemical events in the cell cycle because of its natural synchrony of endomitosis of the plasmodium. Ducommun *et al* found that cdc2 histone H1 kinase activity of *P. polycephalum* variated during cell cycle, peaking in metaphase and dropping very abruptly afterwards[14]. Shipley and Sauer identified a homolog of $p34^{cdc2}$ in *P. polycephalum* and suggested that the homolog was only located in cytoplasm[15]. We previously found, by using monoclonal antibodies against the nonconserved C terminus of $p34^{cdc2}$, that a $p34^{cdc2}$-like protein of *P. polycephalum* situated in both the nucleai and cytoplasm[16]. However,

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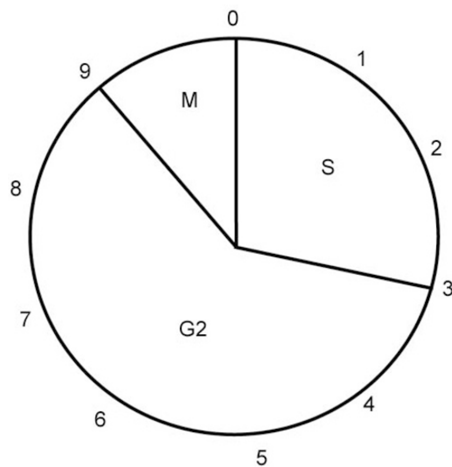

Fig 1. The cell cycle of *Physarum polycephalum.*

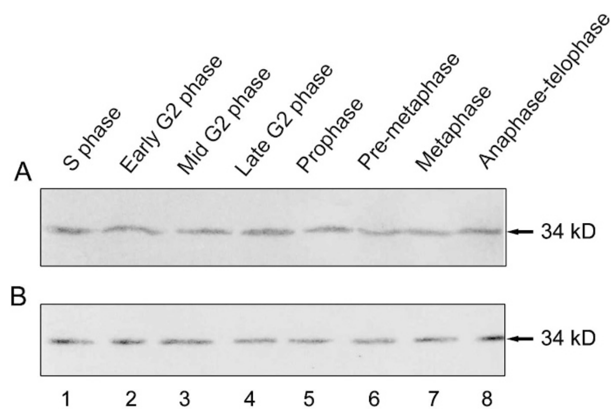

Fig 2. Western blot results of the plasmodia and nuclei proteins at different phases of the cell cycle of *Physarum polycephalum*. (**A**) plasmodia; (**B**) nuclei. A positive band at about 34 kD was detected in plasmodia (A) and nuclei (B) at each phase. The expression of the plasmodia and nuclei specimens at the different phases was consistent.

more evidence is needed to elucidate where PSTAIRE CDKs localize in the cell, and what kind of roles they play during the mitosis in *P. ploycephalum*. In the present study, we carried out Western blot, immunoelectron microscopy and antibody treatment with an anti-PSTAIRE monoclonal antibody to explore the questions above.

MATRIALS AND METHODS

Materials

Strain TU_{291} of *Physarum polycephalum*, was a gift from Dr. Philipe Albert, Cytobiology laboratory of Reims University, France.

Culture methods

Suspended and synchronous culture methods of *P. polycephalum* were referred to Denial and Baldwin[17].

Preparation of plasmodial and nuclear specimens

The cell cycle of *P. polycephalum* consists of S, G2, and M phase, and lacks a G1 phase. The M/S boundary is supposed to be 0 h, and S phase lasts about 3 h, from 0 to 3rd h, G2 phase lasts about 6 h, from 3rd to 9th h, and M phase lasts about 1 h, from 9th to 10th h (Fig 1)[18]. By observing the synchronous macroplasmodia under a light microscope, the specimens of S phase (1.5^{st} h), early G2 phase (4th h), mid G2 phase (6th h), late G2 phase (8th h), prophase (9th h), pre-metaphase, metaphase, and ana-telophase were collected. One half of the plasmodial specimens were directly used for biochemical analyses, and the other half were placed in a pre-cooled solution of nuclei isolation for preparing nuclei specimens according to Mohberg *et al*[19].

SDS-PAGE and Western blot

The nuclei specimens at different phases were dissolved in a sample buffer (100 mM Tris·HCl pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromphenol blue, 20% glycerol) at a dilution of 1:1 (in volume). The plasmodia specimens at different phases were dissolved in the same buffer at a ratio of 0.2 g in wet weight /ml. 30 µl samples lyses per lane were applied to SDS-PAGE using a 10% polyacrylamide gel. Gels were stained with Coomassie brilliant blue.

For Western blot analysis, the proteins were transferred to the nitrocellulose (NC) membranes after electrophoresis. The membrane was blocked for 1 h in a blocking buffer (2% non-fat dry milk in PBS buffer, pH 7.4), and then incubated with anti-PSTAIRE monoclonal antibody (Sigma No. P7962, at a dilution 1:4000 in the blocking buffer) at room temperature for 2 h, washed three times in PBST (0.2% Tween-20 in PBS buffer, pH 7.4, each for 10 min), incubated with a horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG secondary antibody (Chinese Biotech Company, at a dilution of 1:1000 in the blocking buffer) at room temperature for 2 h, then washed three times in PBST (each for 10 min), and stained with diaminobenzidine (DAB).

Immunoelectron microscopy labelling with colloidal gold

By observing the synchronous macroplasmodia under a light microscope, the specimens of S phase, G2 phase, prophase, metaphase and ana-telophase were collected. The specimens were fixed in a mixture of 2.5% glutaraldehyde and 4% paraformaldehyde for 2 h, dehydrated in a grading series of ethanol, and embedded in Lowicryl K4M resin. Sections were cut on a Reichert-Jung ultromicrotome at a thickness of 60-80 nm, disposed in PBSTT (0.05% TritonX-100 and 0.05% Tween-20 in PBS buffer, pH 7.4) at room temperature for 5 min, blocked with 1% BSA (in PBSTT) for 10 min, and then washed in PBS. After that, the sections were incubated with the anti-PSTAIRE monoclonal antibody at a 1:400 dilution in PBS at room temperature for 1 h, washed in PBS and incubated with 10 nm protein A-colloidal gold (Sigma No. P1039) at a 1:25 dilution in PBS at room temperature for 45 min, washed in PBS and double distilled water, stained with 5% uranyl acetate for 15 min. After thoroughly washed in double distilled water, the sections were air dried, observed and photographed at 75 kv with a Hitachi-600 transmission electron

Tab 1. Density of colloidal gold particles in specimens of *P. polycephalum* (Number of particles /μm^2)*

Areas		Number of specimens					M ± SD
		1	2	3	4	5	
Interphase	Chromatin	42.35	40.01	43.21	42.40	41.89	41.97 ± 0.53
	Nucleoli	39.94	41.15	39.89	42.01	41.10	40.82 ± 0.40
	Nucleoplasm	14.31	12.40	13.18	12.40	13.41	13.14 ± 0.36
	Nuclei	24.97	23.98	24.59	24.89	24.99	24.68 ± 0.19
	Cytoplasm	26.15	28.28	27.54	27.55	26.13	27.13 ± 0.43
	Plasmodia	25.29	25.79	25.98	26.01	25.43	25.70 ± 0.15
Prophase	Chromosome	40.66	42.31	41.50	43.01	42.59	42.01 ± 0.42
	Nucleoli	40.37	40.21	40.25	41.09	40.12	40.41 ± 0.18
	Nucleoplasm	5.59	2.10	6.30	11.40	10.79	7.26 ± 1.73
	Nuclei	25.95	23.98	24.32	24.65	24.21	24.62 ± 0.36
	Cytoplasm	25.86	27.94	28.28	27.55	28.01	27.53 ± 0.43
	Plasmodia	25.87	25.65	25.98	26.01	25.75	25.85 ± 0.07
Metaphase	Chromosome	42.01	40.89	42.13	41.75	41.54	41.66 ± 0.22
	Nucleoplasm	19.98	20.90	19.14	20.42	19.81	20.05 ± 0.30
	Nuclei	25.03	25.12	24.56	24.78	24.96	24.89 ± 0.10
	Cytoplasm	27.65	24.50	28.22	28.09	27.31	27.15 ± 0.68
	Plasmodia	25.85	24.98	25.74	25.80	25.65	25.60 ± 0.16
Control		0.79	1.68	1.79	1.88	2.14	1.66 ± 0.23

* The specimens were embedded in Lowicryl K4M, labeled with anti-PSTAIRE monoclonal antibody and protein A-colloidal gold, observed and photographed with H-600-2 electron microscope. The density of gold particles was obtained by analyses and calculations with IBAS image processing system. For the control group, the labeling with anti-PSTAIRE monoclonal antibody was omitted.

microscope. The density of gold particles was obtained by analyses and calculations with IBAS image processing system.

The samples omitted the treatment of the primary antibody served as controls and were run concurrently with each experiment.

Antibody treatment

The synchronous macroplasmodia were treated at early S phase (30 min after the last metaphase) and at early G2 phase (about 4th h). One half of the synchronous plasmodia was continuously cultured in the conventional MSD media as controls, the other half was transferred and cultured in MSD media containing the anti-PASTIRE antibody (at a 1:4000 dilution in MSD). The cell cycle of the control and the treated plasmodia were observed monitored by light microscopy. The slides were prepared at different points of the cell cycle.

RESULTS

Western blot analysis of plasmodia and nuclei at different phases of the cell cycle

The result of Western blot showed that only one specific, positive band was detected at about 34kD in both plasmodia (Fig 2A) and nuclei specimens (Fig 2B) of S phase, early G2 phase, mid G2 phase, late G2 phase, prophase, pre-metaphase, metaphase, and anaphase-telophase, indicating that *P. polycephalum* contained a 34 kD PSTAIRE CDK-like protein and its expression in both plasmodia and nuclei remained consistent through the cell cycle.

Immunoelectron microscopic localization of PSTAIRE CDK-like protein

Immunoelectron microscopy showed that the density of the colloidal gold particles of the labeled specimens was significantly higher than that of the control (Tab 1), indicating that the immunolabelling system used in the present study was specific and the gold particles on the specimens marked the position of the PSTAIRE CDK-like protein.

Few gold particles were found on the control specimens (Fig 3A). In labeled specimens, there were a lot of gold particles in the cytoplasm and nuclei (Figs 3B-D). In the interphase nucleus, gold particles were mainly distributed in nucleoli and chromatin areas (Fig 3B). In prophase nucleus, gold particles were mainly distributed in nucleoli and chromosomes (Fig 3C). In the metaphase nuclei, a large number of gold particles were concentrated on chromosomes (Fig 3D). The average density of gold particles in interphase, prophase and metaphase plasmodia (cell) (25.70/μm^2, 25.85 and 25.60/μm^2, respectively) was the same on the whole; and that of interphase, prophase and

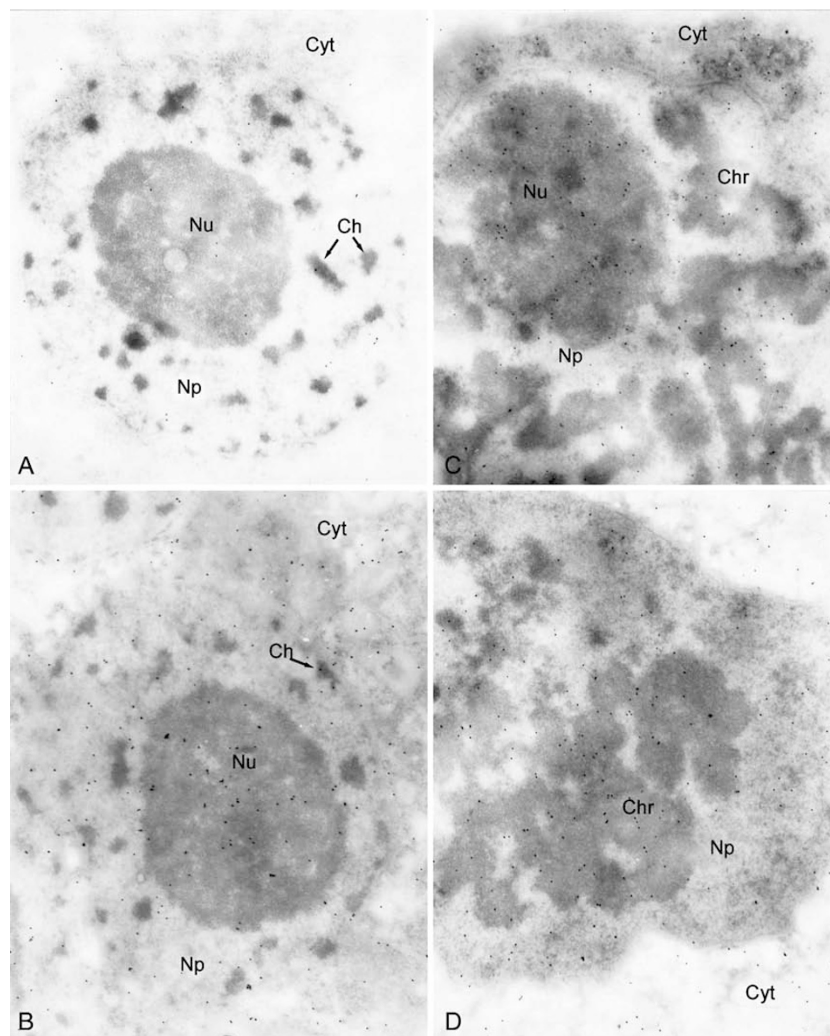

Fig 3. Electron microscopic images of the plasmodia of *Physarum polycephalum* labeled with anti-PASTIRE antibody and protein A-colloidal gold. (**A**) Control group, in which the labeling with anti-PSTAIRE monoclonal antibody was omitted. Few gold particles were found on the control specimens. (**B**) Interphase (G2 phase) plasmodia; (**C**) Prophase, (**D**) Metaphase. Many gold particles were distributed in the cytoplasm and nuclei (B, C, D). In the nuclei, gold particles were mainly cocated in nucleoli (B, C) and the chromatin (B) or chromosomes (C, D). Cyt: cytoplasm, Nu: nucleoli, Np: nucleoplasm, Chr: chromosomes, Ch: chromatin. ×25 000

metaphase nuclei (24.68/μm^2, 24.62 and 24.89/μm^2, respectively) was also about the same (Tab 1).

Retardation of the cell cycle by anti-PASTIRE antibody

The control groups were going on normal endomitosis (Fig 4A-D). In S phase, the chromatin and nucleolar contents mixed together in small nuclei (Fig 4A). In early prophase, the nucleolus was to the side of the nucleus (Fig 4B). In metaphase, chromosomes clusters were aligned on equatorial plate (Fig 4C). In telophase, sister chromatids migrated to different poles, and then two daughter nuclei formed (Fig 4D). However, the cell cycle of the anti-PASTIRE antibody-treated specimens was somewhat inhibited or retarded (Figs 4E-H). In the specimens treated at early S phase, the cells were arrested in S phase and cell division was completely prevented (Fig 4E). While in the specimens treated at early G2 phase, the mitotic onset of the cell cycle was blocked for about 1h, and the mitosis progression was retarded (Figs 4F-H). About 1h after the control plasmodia began their M phase, the treated group just entered early prophase (Fig 4F).

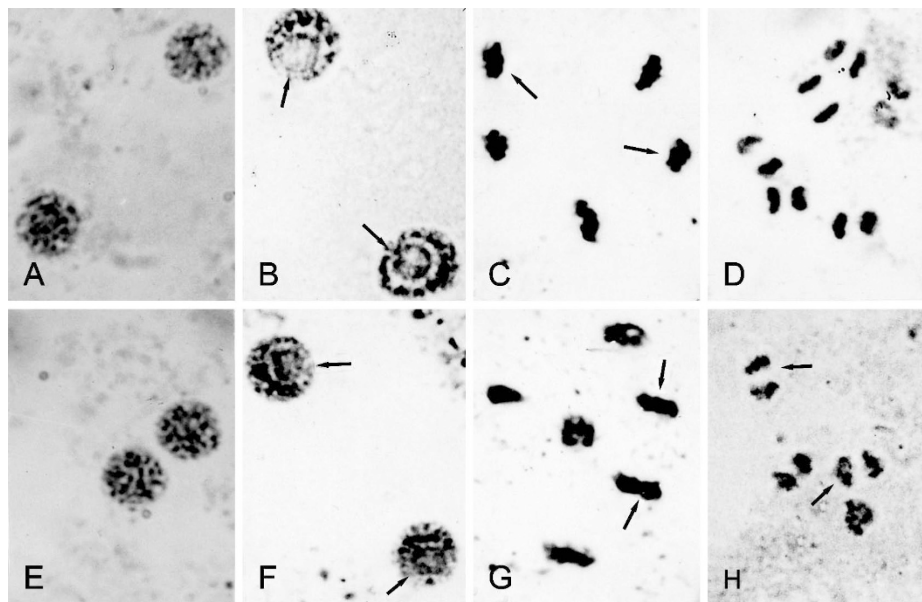

Fig 4. Light microscopic photos showing effects of anti-PSTAIRE antibody treatment upon the cell cycle of *Physarum polycephalum*. (**A-D)**. Nuclei went on normal mitosis. (**E-H**) Antibody treated specimens. (**E**) Nuclei treated with antibody at early S phase and arrested in S phase. (**F-H**) Nuclei treated at early G2 phase. The mitotic onset of the cell cycle was blocked for about 1 h, and the mitosis progression was retarded. ×2000

When the control plasmodia were in the next cell cycle for about 50 min, the treated plasmodia entered their first metaphase (Fig 4G). When the control was in the next cell cycle for about 60 min, the treated just entered their first telophase (Fig 4H).

DISCUSSION

CDKs are the central components of eukaryotic cell cycle regulation[1]. In yeast, a single CDK (cdc2 in *Schizosaccharomycds pombe* or CDC28 in *Saccharomyces cerevisiae*) governs both the G1/S and G2/M transitions. It contains the PSTAIRE sequence in its cyclin binding domain and binds to different cyclins at different cell cycle stages[1]. In animals, PSTAIRE CDKs include three types, i.e. cdc2/CDK1, CDK2 and CDK3[20-22]. In plants, PSTAIRE CDKs have been identified in several species, such as *Arabidopsis* (CDC2a), maize (cdc2ZmA), rice (cdc2Os-1 and cdc2Os-2), alfalfa (cdc2MsA and cdc2-MsB) and *Fucus*[9, 10]. In *P. polycephalum*, a lower plant often used as an experimental model, Ducommun *et al* detected cdc2-related activity (histone H1 kinase activity) [14], and Shipley and Sauer found a 34 kD cdc2 homolog by using a peptide antibody directed against a consensus conserved sequence near the N-terminal end of $p34^{cdc2}$ [15]. In our previous studies, using specific monoclonal antibodies against the nonconserved C terminus of $p34^{cdc2}$, we found a $p34^{cdc2}$ -like protein in *P. polycephalum*[16]. In this paper, biochemical and immunoelectron microscopic evidence indicated that *P. polycephalum* contains a PSTAIRE CDK-like protein or CDK A-like protein. This PSTAIRE CDK-like protein and the previously reported $p34^{cdc2}$ -like protein varied little in their molecular weight, expression and subcellular location during the cell cycle, and the cell cycle were retarded by the treatment of the anti-PASTIRE antibody and anti-$p34^{cdc2}$ antibody[16], strongly suggesting that they may be the same protein. The PSTAIRE CDK-like protein reported in this paper and the 34 kD cdc2 homolog identified by Shipley and Sauer [15] share common features, such as molecular weight and content consistence during the cell cycle, suggesting that they also might be the same protein.

A steadily accumulating body of evidence points to the control of subcellular localization of a number of essential proteins, particularly CDC2, cyclinB, cyclinD, CDC25, and CDC6, as an important mechanism of cell cycle control in eukaryote[24]. When assayed by indirect immunofluorescence, PSTAIRE-like CDKs in higher plants (maize, alfalfa, and *Arabidopsis)* were predominantly found in the interphase and early prophase nucleus, and to a lesser extent in the cytoplasm[11-13]. During mitosis, the PSTAIRE CDKs have been found in association with a number of cytoskeletal structures such as preprophase band, spindle,

and phragmoplast[11-13]. They also transiently interacted with the chromosomes at the metaphase-anaphase transition in alfalfa[12], but apparently not in maize[13]. In the present study, Western blot and *in situ* immunoelectron microscopy indicated that the PSTAIRE CDK-like protein of *P. polycephalum* was in both nuclei and cytoplasm throughout the cell cycle, and the protein was mainly associated with nucleoli and chromosomes in the nuclei. The subcellular localization of the PSTAIRE CDK-like protein reported in this paper was different from what was reported in the higher plants[11-13]. The difference possibly results from the differences in the methods and materials used. While the immunofluorescence microscopy was used to localize PSTAIRE CDKs in the previous studies [11-13], we applied immunoelectron microscopy to analyze the subcellular location of the PSTAIRE CDK-like protein at ultrastructural level. So our results may demonstrate the location of the protein more precisely. In addition, *P. polycephalum* is a lower plant. The subcellular localization of PSTAIRE CDK-like proteins in lower plants may be different from the higher ones.

The results of microinjection of anti-p34^{cdc2} antibodies [25] or p34^{cdc2} kinase[26] into rat fibroblasts revealed that PSTAIRE CDKs play important roles in animal cell cycle regulation. In plants, however, studies on the function of PSTAIRE CDKs in cell cycle regulation are still rare[1, 7-10]. Although Hush *et al*. injected active CDK complexes of metaphase plant cells into *Tradescantia* stamen hair cells and found rapid disintegration of the preprophase band, nuclear envelope breakdown, and chromosome condensation, the composition of the complexes used in the study was unknown[27]. Recently, Corellou *et al* injected an anti-PSTAIRE antibody into *Fucus* zygotes and found that when injected early (1 h after fertilization), anti-PSTAIRE antibody completely prevented cell division and inhibited germination, while when injected at 8 h after fertilization (i.e. in S phase), the cell divided and germinated normally [10]. In the present study, the mitosis of *P. polycephalum* was arrested in S phase when treated with anti-PSTAIRE antibody in early S phase, and the mitotic onset was blocked for about 1 h when treated with the antibody at early G2 phase. Although the plasmodium of *P. polycephalum* used in the present study was quite different from the *Fucus* zygotes and the response after the antibody treatments showed some differences, our results were consistent with the results of Corellou *et al*. in that PSTAIRE CDKs have a direct bearing on the mitosis of plant cells.

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