分裂酵母の休眠からの復帰過程におけるシグナル伝達と細胞質流動性の研究

Studies on the signal transduction and the cytoplasmic fluidity during dormancy breaking in fission yeast

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Introduction

Cellular dormancy is a physiological state, in which cells are non-proliferative under stress conditions [1–3]. To study cellular dormancy, fission yeast, *Schizosaccharomyces pombe*, is one of the valuable model organisms, because nitrogen depletion allows cells to enter a dormant state and form spores [4]. Spore formation renders the cells resistant to several stresses such as nutrient starvation, heat shock, and organic solvents [5–7]. Glucose refeeding induces dormancy break of spores, resuming cell growth and proliferation [8]. This process is called germination.

Sporulation-deficient mutants in *S. pombe* had been isolated by forward genetics in the 1960 s and then the sporulation-specific genes had been cloned in the 1980 s [9–11]. In early 2000, gene expression patterns during sporulation had been analyzed by using DNA microarrays [12]. Thus, protein or gene functions involved in sporulation have hitherto been investigated. In contrast to sporulation, the molecular mechanisms underlying germination are poorly understood because of the technical difficulties. For example, fission yeast spores germinate asynchronously, making it difficult to implement omics-based methods using spores populations [1]. In addition, gene expression patterns and morphogenesis during germination have been investigated based on single-cell RNA-seq and live-cell imaging, respectively [1,13]. However, it is still unclear what happens in the earlier stage of germination before gene expression and morphogenesis, and what causes the asynchronous germination of fission yeast spores.

In budding yeast, *Saccharomyces cerevisiae*, it has been reported that stress tolerance under starvation or stress conditions requires a decrease in cytoplasmic fluidity [14]. Trehalose, a disaccharide, is known to reduce the fluidity in vegetative budding yeast cells, and accumulate during sporulation in fission yeast [15,16]. Furthermore, it has been known that the glucose-stimulated cAMP-PKA pathway is required for germination initiation in fission yeast spores [4]. These evidences suggest that cytoplasmic fluidity in fission yeast is reduced by trehalose accumulation, and that the cAMP-PKA pathway is involved in glucose-induced fluidization of cytoplasm through regulation of trehalose degradation. In this study, I aimed to elucidate the relationship between cytoplasmic fluidity and germination in fission yeast spores, and the possible role of the cAMP-PKA signaling in asynchronous germination by live-cell imaging.

Materials and Methods

Fission yeast strain and culture

The growth medium, sporulation medium, and other techniques for fission yeast were based on the protocol described previously [17]. The transformation protocol was modified from that of Suga and Hatakeyama [18]. For fluorescence microscope imaging, the fission yeast cells were concentrated by centrifugation at 860 g, mounted on a slide glass, and sealed by a cover glass (Matsunami).

Live-cell fluorescence imaging of fission yeast cells

Cells were imaged with an IX83 inverted microscope (Olympus) equipped with an sCMOS camera (ORCA-Fusion BT; Hamamatsu Photonics), an oil objective lens (UPLXAPO 100×, NA = 1.45, WD = 0.13 mm; or UPLXAPO 60× NA = 1.42, WD = 0.15 mm; Olympus) and a spinning disk confocal unit (CSU-W1; Yokogawa Electric Corporation). The excitation laser and fluorescence filter settings were as follows: Excitation laser, 488 nm and 640 nm for 40 nm-GEM (T-Sapphire) and iRFP, respectively; excitation dichroic mirror, DM405/488/561/640; emission filters, 525/50 for 40 nm-GEM (T-Sapphire), and 685/40 for iRFP (Yokogawa Electric).

Quantification of trehalose amount

In sample preparation, vegetative cells or spores are counted by using a cell counter Moxi Go II (Orflo, Technologies). Cells were washed with 1 mL of glucose-free YEA medium and added with 100 μL of boil water. After vigorous vortexing, samples were boiled for 15 minutes and then centrifuged. The supernatant was collected into new tubes (the first extraction). The pellet was added with 100 μL of boil water and vortexed for 5 minutes. The supernatant was collected into the tubes (the second extraction).

Trehalose was quantified by using the Trehalose Assay Kit (K-TREH, Megazyme) according to manufacturer's instructions and previously reported methods [15]. Absorbance at 340 nm was measured by using Thermo Scientific Multiskan FC (Thermo Fisher Scientific).

Imaging data analysis

Fluorescence imaging data were analyzed and quantified using Fiji/ImageJ (https://fiji.sc/). The background was subtracted using the rolling ball method. For the quantification of iRFP signal intensity in fission yeast cells, appropriate regions of interest (ROIs) were manually selected, and mean intensities in ROIs were measured. For the tracking of 40 nm-GEM, the Mosaic suite, a Fiji/ImageJ plugin, was used with the following parameters: Radius = 3, Cutoff = 0, Per/Abs = 0.2 ~ 0.5, Link Range = 1, Displacement = 6, Dynamics; Brownian. Data visualization and graph creation were performed using Python 3.10 with Numpy 1.21.3, Pandas 1.3.4, Matplotlib 3.4.3, and Seaborn 0.11.2 modules.

Results and Discussion

First, I quantified the cytoplasmic fluidity in fission yeast spores during germination by using a genetically encoded multimeric nanoparticle (GEM) with a diameter of 40 nm [19]. The GEMs are homomultimeric scaffolds that self-assemble into stable particles. By tracking single fluorescent particles, I measured the effective diffusion coefficients of GEMs in vegetative cells and spores. I found that the diffusion coefficient in spores becomes approximately 30-fold smaller than that in vegetative cells. Further, such a solid-like cytoplasm of the spores was rapidly fluidized within an hour by glucose-induced germination. These results demonstrated that the spore cytoplasm is in a solid-like state, and cytoplasmic fluidity rapidly increases in spores during germination. To investigate the mechanisms underlying the increase in the fluidity of spores, I focused on the glucose-stimulated cAMP-PKA pathway, because glucose induces spore germination through the cAMP-PKA pathway [4]. The depletion of genes associated with the cAMP-PKA pathway prevented cytoplasm fluidization and spore germination induced by glucose stimulation. This result strongly suggests that the cAMP-PKA pathway is required for germination and cytoplasmic fluidization. Next, I examined possible links between the cAMP-PKA pathway and trehalose synthesis/degradation. The GEMs imaging data demonstrated that the glucose-induced germination causes rapid trehalose degradation at the same time as increased cytoplasmic fluidity. Disruption of the trehalose-degrading gene, *ntp1*, inhibited cytoplasmic fluidization and spore germination, indicating that cytoplasmic fluidization needs trehalose degradation for germination. Furthermore, *pka1*-deficient mutant spores did not show any trehalose degradation. These data revealed that glucose-stimulated germination requires the activation of the cAMP-PKA pathway, followed by cytoplasmic fluidization via Ntp1-mediated trehalose degradation.

Next, to clarify the role of the cAMP-PKA pathway in the asynchronous germination, I established live-cell imaging systems to visualize and manipulate the PKA activity. Because of the lack of tools to visualize the PKA activity in yeasts, I developed a PKA biosensor, called spPKA-KTR, which is based on the principle of kinase translocation reporter (KTR) [20]. spPKA-KTR shuttles between the nucleus and cytoplasm depending on its phosphorylation by PKA, and thus the subcellular localization of spPKA-KTR reflects PKA activity. As expected, glucose stimulation in vegetative cells induced translocation of spPKA-KTR from the nucleus to the cytoplasm, indicating PKA activation. To quantify the subcellular localization of spPKA-KTR, I tested a near-infrared fluorescent protein iRFP fused with nuclear localization signals (NLS-iRFP-NLS) as a nuclear marker [21]. Unexpectedly, NLS-iRFP-NLS did not fluoresce at all in fission yeast. To overcome this issue, I tried to develop a method for iRFP imaging in fission yeast. In a series of experiments, I found that phycocyanobilin (PCB) functions as a chromophore for iRFP and enhances its fluorescence more than biliverdin (BV), which is a conventional chromophore for iRFP. The addition of purified PCB to cells expressing NLS-iRFP-NLS outperformed the addition of BV regarding iRFP fluorescence intensity.

Further, I applied a PCB biosynthesis system, SynPCB, to iRFP imaging in fission yeast [22], allowing NLS-iRFP-NLS fluorescence imaging without adding purified PCB. To manipulate cAMP level and PKA activity, I introduced a photoactivated adenylate cyclase bPAC into fission yeast [23]. Combining spPKA-KTR with bPAC enabled to visualization and manipulation of intracellular PKA activity in vegetative fission yeast cells. However, unfortunately, spPKA-KTR could not function in spores due to its aggregation.

In summary, I found that cytoplasmic fluidization plays an essential role in germinating of fission yeast spores through trehalose degradation via the cAMP-PKA pathway. Because macromolecules such as RNA polymerase and ribosomes are comparable in size to GEMs [24,25], their diffusion could be suppressed in spores. Therefore, it is suggested that spores maintain dormancy by decreasing transcription and/or translation, and cytoplasmic fluidization triggers dormancy breaking and germination initiation through resuming RNA and/or protein synthesis. PKA imaging system with spPKA-KTR and bPAC would provide an experimental basis for investigating PKA activity dynamics in fission yeast.

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