論文内容の要旨

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論文題目

Studies on Stress Granules in *Aspergillus oryzae*
（麹菌 *Aspergillus oryzae* におけるストレス顆粒に関する研究）

Introduction

Microorganisms are constantly challenged by ever-changing variables in their environment. The ability to sense environmental stimuli, including stress, activate signal transduction, and mount appropriate acute and adaptive responses is crucial for eukaryotic cell survival. Adaptation requires physiological and metabolic changes, and is ultimately achieved through the regulation of gene expression. Traditionally, transcriptional regulation has been regarded as the major determinant of gene expression. However, accumulating evidence indicates that posttranscriptional modulation of mRNA stability and translation plays a key role in the control of gene expression and provides greater plasticity, allowing cells to immediately adjust protein synthesis in response to changes in the environment. Recent studies have demonstrated that one aspect of this modulation involves the remodeling of mRNAs translated from polysomes into non-translating messenger ribonucleoproteins (mRNPs), which accumulate in discrete cytoplasmic foci known as stress granules and processing bodies (P-bodies).

The common feature of environmental stress response in eukaryotic cells is the global translation inhibition, in which cells shut down protein synthesis to conserve anabolic energy and initiate a reconfiguration of gene expression. The stalled preinitiation complexes, together with their associated mRNAs, are routed into stress granules. However, a subset of mRNAs required for cell survival under stress conditions are not delivered to stress granules but stabilized and preferentially translated in the cytoplasm. The function of stress granules is not fully understood; however, they have been implicated in the posttranscriptional regulation processes, such as translational repression, mRNA storage, and cellular signal transduction.

Stress granules are conserved in eukaryotes and have been studied extensively in yeast and mammalian cells. However, stress granules in filamentous fungi, including *Aspergillus oryzae*, have not yet to be defined. For this reason, the present work was to study stress granules in *A. oryzae*.

Chapter 1. Cytoplasmic mRNP granules in *A. oryzae*

The poly(A)-binding protein Pab1p, which is involved in the translational regulation and stability of mRNAs, is one of the most reliable and easily visualized components of stress granules in the yeast *Saccharomyces cerevisiae*. A homolog of the *S. cerevisiae PAB1* gene was found in the *A. oryzae* genome database and designated as *Aopab1* (AO090003000927). To monitor stress granules by fluorescence microscopy, an AoPab1-EGFP fusion protein was expressed in *A. oryzae* under control of the *amyB* promoter as a stress granule marker. Global translational arrest is a common environmental stress response in eukaryotes, and the inhibition of translation initiation leads to the formation of stress granules. As a first step in characterizing stress granules in *A. oryzae*, several external stimuli were used to assess the induction of stress granules in cells. Under normal growth conditions, AoPab1-EGFP was dispersed throughout the cytoplasm. Exposing cells to stress, including heat stress (45°C, 10 min), cold stress (4°C, 30 min), glucose deprivation (10 min), ER stress (10 mM DTT, 60 min), osmotic stress (1.2 M sorbitol, 30 min), and oxidative stress (2 mM H_2O_2, 30 min), led to an induction of stress granules, as judged by the accumulation of AoPab1-EGFP as cytoplasmic foci. In eukaryotic cells, non-translating mRNAs also accumulate in P-bodies, which contain a conserved core of proteins involved in translational repression and mRNA degradation. To elucidate the spatial and functional links of stress granules and P-bodies, a strain co-expressing the P-bodies marker AoDcp2-EGFP and AoPab1-mDsRed was examined after being exposed to 45°C for 10 min. Under heat stress, stress granules were found to colocalize with P-bodies in *A. oryzae*. Finally, to investigate the functional importance of stress granule formation on cell survival against stress, an *A. oryzae* homolog of a well-known stress granule marker important for stress granule formation, *Aopub1*, was disrupted and cultured on PD plates containing 10 mM DTT, 2 mM H_2O_2, or 1.2 M sorbitol. The phenotype of the *Aopub1* disruptant was characterized by a slower growth rate, and a severe impairment in conidia formation. The growth retardation of the *Aopub1* disruptant was more severe under all examined stress conditions. Taken together, these results indicate that the formation of stress granules is a general phenomenon in response to external stress, which occurs also in *A. oryzae*.

Chapter 2. Identification of AoSO as a novel component of stress granules upon heat stress in *A. oryzae*

The mycelia of filamentous fungi consist of a network of interconnected hyphae, which are compartmentalized by septa. Septa contain a central pore that allows the movement of cytoplasm and organelles between adjacent hyphae for direct communication and coordination. However, in response to hyphal damage and stressful environmental conditions, cells defend against cytoplasmic loss by the rapid

occlusion of septal pores. It has been shown previously that an *A. oryzae* homolog of *Neurospora crassa* SO (SOFT) protein AoSO accumulates at the septal pore when cells are exposed to various stresses. The stress-induced accumulation behavior of AoSO suggests that it may interact with stress granules. To investigate this possibility, a strain co-expressing AoSO-EGFP and AoPab1-mDsRed was used to examine the relative localizations of AoSO and stress granules in cells exposed to heat stress. AoPab1-mDsRed did not accumulate at the septal pore in cells exposed to heat stress. However, in cells exposed to heat stress, AoSO-EGFP also accumulated as cytoplasmic foci, which colocalized with stress granules labeled with AoPab1-mDsRed at the hyphal tip. The physical interaction between AoSO-EGFP and AoPab1-3HA was confirmed by co-immunoprecipitation, although the association between AoSO-EGFP and AoPab1-3HA was not induced or increased after exposure to heat stress. To clarify if the aggregation of AoSO requires the presence of non-translating mRNAs, cycloheximide, which blocks translational elongation and traps mRNAs in polysomes, was used to deplete the pool of non-translating mRNAs. The heat stress-induced formation of cytoplasmic AoSO foci at the hyphal tip was greatly impaired by cycloheximide, suggesting that cytoplasmic AoSO foci require a pool of free mRNAs for their aggregation. However, cycloheximide did not affect the accumulation of AoSO at the septal pore. To gain a better understanding of the role of AoSO in stress granules, the effect of *Aoso* deletion on stress granule formation was examined. Heat stress-induced formation of stress granules was slightly impaired in the *Aoso*-deletion strain. The effect of *Aoso* deletion on stress granules was further evaluated by measuring the distance between the hyphal tip and stress granules. The distribution of the largest stress granules labeled with AoPab1-EGFP was less concentrated and more distant from the hyphal tip in the *Aoso*-deletion strain. Taken together, the results suggest that AoSO is a novel component of mRNP granules in *A. oryzae*, which influences the formation and localization of stress granules in cells exposed to heat stress.

Chapter 3. Participation of autophagy in the clearance of stress granules under sustained heat stress

The formation of stress granules in response to stress is a conserved phenomenon in eukaryotes, but little is known about the fate of stress granules. Autophagy is a conserved and tightly regulated process in eukaryotic cells for the bulk degradation of cytoplasmic components in the vacuole or lysosome to maintain cellular homeostasis. In *A. oryzae*, stress granules were often found to dock with vacuoles, suggesting a possibility that under sustained stress condition, stress granules are degraded by autophagy. To clarify it, a strain co-expressing the autophagosome marker EGFP-AoAtg8 and AoPab1-mDsRed was used to examine their relative

localizations, and the fusion proteins were colocalized in cells exposed to heat stress. Additionally, after exposure to heat stress, protein amounts of stress granule component AoPab1-3HA was decreased in a time-dependent manner. However, in an autophagy-defective strain, *Aoatg8* disruptant, the degradation of AoPab1-3HA induced by heat stress was greatly inhibited. These results indicate that under sustained heat stress, the degradation of stress granule component is at least partially mediated through autophagy.

Chapter 4. The posttranslational modification of stress granule component AoPab1 in response to heat stress

Poly(A)-binding protein is a central regulator of mRNA translation and stability and is a core component of stress granules, suggesting that complex modulations are required to coordinate its multi-functions and rapid response to stress. Post-translational modification (PTM) is a crucial mechanism to regulate the functions of many eukaryotic proteins. To elucidate whether AoPab1 was post-translationally modified, lysates from unstressed cells or cells exposed to heat stress for 10 or 30 min were analyzed by two-dimensional SDS/PAGE. AoPab1-3HA was detected as a continuing band, indicating multiple PTM isoforms. In addition to a portion of isoforms with higher pI (basic isoforms), highly acidic isoforms appeared in cells exposed to heat stress for 10 min, and were not observed after exposure of heat stress for 30 min. Exposure to heat stress for 30 min induced a broad distribution of AoPab1 isoforms from pH 3-10 with highly basic species. To identify the PTMs in AoPabl, AoPabl-3HA was immunoprecipitated and analyzed using LC-MS/MS. Methylation, acetylation, and phosphorylation were identified at several residues throughout all the functional domains of AoPab1, with an exception of Q/G/P-rich region. Exposure to heat stress resulted in changes in PTMs of AoPab1. These results indicate that AoPab1 is subject to extensively post-translationaly modified and regulated dynamically in response to heat stress.

Conclusion

A. oryzae is an industrially important species for hetrogenous protein production. However, low yield is still a major bottleneck for hetrologous protein production, even many genetic approaches have been used to improve the productivity. The present study provides an integrated perspective on eukaryotic stress response. Understanding of post-trancriptional regulation of gene expression on mRNA modulation may provide new insights for industrial application in the near future.

Reference

H-T Huang, J Maruyama, and K Kitamoto. *Aspergillus oryzae* AoSO is a novel component of stress granules upon heat stress in filamentous fungi. PLOS ONE 8(8): e72209, 2013.