論 文 概 要

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Post-transcriptional regulation of gene expression by the RNA-binding protein Puf5 and the Ccr4-Not complex in yeast

（酵母におけるRNA結合タンパク質Puf5とCcr4−Not複合体による遺伝子発現の転写後制御）

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Purposes

The Ccr4-Not complex is an essential multi-subunit complex that is conserved from yeast to human and plays an important role in regulation of mRNA decay [1, 2]. Puf5 is a broad mRNA regulator that interacts with more than 1,000 mRNAs and is involved in multiple cellular processes [3-6]. It has been revealed that Puf5 binds to the recognition sequences in the 3'UTR of their target transcripts and recruits the Ccr4-Not complex through the interaction with Pop2 subunit [7, 8]. However, the physiological roles of Puf5 and Pop2 as well as how these two proteins are regulated remain unknown. Therefore, I aim to analyze the regulation of Puf5, in concert with Ccr4 and Pop2 deadenylases, on *LRG1* expression in CWI pathway. In addition, in order to further understand how Puf5 and Pop2 are regulated by glucose availability, I examined the effect of glucose-regulated phosphorylation on the functions of these two proteins.

Materials and methods

1. Strains, plasmids, and general methods

Escherichia coli DH5α was used for DNA manipulations. *Saccharomyces cerevisiae* strains used in this study were derived from W303 [5]. General procedures were performed as described previously [9]. Gene disruption and insertion were performed using PCR-based gene replacement, as described previously [10, 11].

2. Western blot analysis

Extracts were subjected to SDS-PAGE on acrylamide gels followed by electroblotting onto an Immobilon membrane. Phos-tag was added to a mix of SDS-PAGE gel when required. The membrane was incubated with antibody to detect protein.

3. RNA isolation, RT-qPCR, and microarray analysis

First strands of cDNA were generated from isolated RNA. The cDNAs of *LRG1*, *HSP12*, *HSP26*, and *PIR3* were quantitated by a quantitative real-time PCR method.

The microarray analysis was performed by the KURABO Bio-Medical Department using the Affymetrix GeneChip Yeast Genome 2.0 Array.

4. Poly(A) tail length assay

The poly(A) tail length of *LRG1* mRNA was measured by sequencing.

Results

1. Regulation of LRG1 expression by RNA-binding protein Puf5 in the budding yeast cell wall integrity pathway

The PUF RNA-binding protein Puf5 is involved in regulation of cell wall integrity (CWI) pathway in yeast by negatively regulating the expression of *LRG1* mRNA, encoding for GTPase-activating protein for Rho1 small GTPase. Here I further analyzed the effect of Puf5 on *LRG1* expression, together with Ccr4 and Pop2 deadenylases, Dhh1 decapping activator, and other PUF proteins. I found that the growth defect of *puf5*Δ mutant was enhanced by *ccr4*Δ mutation, which was partially suppressed by *LRG1* deletion. Consistently, Lrg1 protein level was much more up-regulated in *ccr4*Δ *puf5*Δ double mutant than in each single mutant. Interestingly, *LRG1* poly(A) tail length was longer in *ccr4*Δ mutant but not in *puf5*Δ mutant. Thus, Puf5 regulates *LRG1* expression independently from Ccr4, although Puf5 recruits the Ccr4-Not deadenylase complex for mRNA destabilization.

Unexpectedly, *puf6*Δ mutation suppressed the growth defect caused by *ccr4*Δ *puf5*Δ mutation. Loss of ribosomal proteins Rpl43a and Rpl43b, the previously identified Puf6 interactors, also suppressed the growth defect of *ccr4*Δ *puf5*Δ mutant. My results suggest that Puf6 is involved in the Ccr4 and Puf5-mediated regulation of cell growth through association with Rpl43a and Rpl43b.

2. RNA-binding protein Puf5 is phosphorylated at multiple sites in both glucose and gly-lac media

I examined the phosphorylation of Puf5 under 2 different conditions, medium containing glucose as a carbon source (glucose medium) and medium containing non-fermentable glycerol and lactate (gly-lac medium) as carbon sources, and observed different mobility shifts of Puf5 in two conditions. My results show that Puf5 is phosphorylated at multiple sites in both glucose and gly-lac media but more significantly in gly-lac medium. Additionally, Puf5 phosphorylation is dependent on two kinases, Snf1 and Pho85, since Puf5 phosphorylation is significantly decreased in *snf1Δ* and *pho85Δ* mutants in both conditions.

Overexpression of *PUF5* efficiently suppressed the growth defect of *puf5Δ* mutant at high temperature, but overexpression of *PUF5* harboring phospho-defective mutations did not, suggesting that the phosphorylation plays critical role on Puf5 function at high temperature. Furthermore, phospho-defective *PUF5* displays stronger repression of *HO* repoter mRNA, providing evidence that Puf5 phosphorylation regulates its function to degrade target mRNAs.

3. *Pop2 phosphorylation at S39 is important for Pop2 to repress the expression of stress response genes, HSP12 and HSP26, upon glucose availability.*

I found that Pop2 is phosphorylated at serine 39 (S39) under unstressed conditions. The dephosphorylation of S39 was occurred within 1 min after glucose depletion, and the addition of glucose to the glucose-deprived culture recovered this phosphorylation, suggesting that Pop2 phosphorylation at S39 is regulated by glucose. Pop2 phosphorylation at S39 is involved in the expression of *HSP12* and *HSP26*, encoding small heat shock proteins. In medium supplemented with glucose, Pop2 might be phosphorylated at S39 by Pho85 kinase to repress the expression of *HSP12* and *HSP26*. Glucose starvation inactivated Pho85, which resulted in the derepression of *HSP12* and *HSP26*. Thus, Pop2 phosphorylation at S39 is important for Pop2 to repress the expression of stress response genes, *HSP12* and *HSP26*, in the presence of glucose. My results reveal that Pho85-dependent phosphorylation of Pop2 is a part of the glucose sensing system in yeast.

Discussion

1. Regulation of LRG1 expression by RNA-binding protein Puf5 in the budding yeast cell wall integrity pathway

Puf5 regulates *LRG1* mRNA in other manner that independent of deadenylation. Moreover, Puf6 is also likely to be involved in cell growth regulation of Ccr4 and Puf5. My study provides valuable insights into the roles of two PUF RNA binding proteins, Puf5 and Puf6, in the growth of yeast cells. However, the molecular mechanism how Puf5 regulates *LRG1* mRNA remains unknown and will be the focus of future studies. Further experiments are also needed to identify other mRNAs regulated by Puf5 in deadenylation independent-mechanisms.

2. RNA-binding protein Puf5 is phosphorylated at multiple sites in both glucose and gly-lac media

Multiple migrations of Puf5 with different patterns in glucose and glucose-deprived conditions were observed; however, the phosphorylation positions of Puf5 in each condition have not been identified exactly. Further experiments are needed to determine precisely Puf5 phosphosite in each condition. Future studies are also required to reveal the regulation that kinases including Snf1 and Pho85 regulate Puf5 function.

3. *Pop2 phosphorylation at S39 is important for Pop2 to repress the expression of stress response genes, HSP12 and HSP26, upon glucose availability.*

Pop2 is phosphorylated in a Pho85-dependent manner and this phosphorylation contributes to the suppression of stress response genes, *HSP12* and *HSP26*. My study provides valuable insights the role of Pop2 in stress responses in yeast. Nevertheless, the molecular mechanism how Pop2 regulates *HSP12* and *HSP26* mRNAs through S39 phosphorylation need to be further analyzed.

Conclusion

The main objectives of my study were to investigate the physiological roles of Puf5 and Pop2 as well as how these proteins are regulated in response to glucose availability. Firstly, my results suggest that Puf5 functions in the CWI pathway by regulating *LRG1* expression in a deadenylase-independent manner, and that Puf6 is involved in the Ccr4 and Puf5-mediated regulation of cell growth through association with Rpl43a and Rpl43b. Secondly, I found that Puf5 is phosphorylated at multiple sites in both glucose and glucose deprivation conditions. Puf5 phosphorylation not only plays important role in the function of this protein at high temperature but also influences its target mRNA degradation. In my third study, I demonstrated that Pop2 phosphorylation at S39, which is dependent on Pho85 kinase, is important for Pop2 to repress the expression of stress response genes, *HSP12* and *HSP26*, upon glucose availability.

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