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学位論文名	Functional redundancy of protein phosphatases, Ptp2p and Msg5p, for calcium stress response in *Saccharomyces cerevisiae* （出芽酵母のカルシウムストレス応答に必要なプロテインホスファターゼPtp2p、Msg5pの機能重複）
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論文内容の要旨

Chapter 1　General Introduction

Reversible phosphorylation of proteins by protein kinase (PKase) and protein phosphatase (PPase) is an essential regulatory mechanism that occurs in eukaryotic cells from humans to yeast. PKase and PPase regulate a variety of cellular processes such as signal transduction, genes expression and cell cycle. In order to understand physiological role of PPases in eukaryotes, we have started constructing a series of *Saccharomyces cerevisiae* disruptants for each of PPase genes (30 out of 32 known at that time except for two essential PPases) and furthermore 435 ppase double disruptants in all possible combinations of 30 ppase single disruptants to study functional redundancy of PPases. Through genetic screening for new phenotype, it was discovered that double disruption of *PTP2* (protein tyrosine phosphatase sub-family) and *MSG5* (dual-specificity protein phosphatase sub-family which dephosphorylates phospho-serine, -threonine, and -tyrosine residues) leads to a calcium sensitive phenotype (Ca^s) whereas single disruption of either *PTP2* or *MSG5* does not, indicating the functional redundancy of these two PPases belonging to different sub-families.

Chapter 2　High Ca^{2+} concentration induces delay of G1-S transition through down-regulation of *CLN2* expression in the Δ*ptp2* Δ*msg5* double disruptant

To investigate whether the Δ*ptp2* Δ*msg5* double disruptant exhibits growth arrest at any specific point of the cell cycle, particularly in the presence of exogenous Ca^{2+}, FACS analysis was conducted. It was found that growth progression of the Δ*ptp2* Δ*msg5* double disruptant slowed down at the G1 to S transition even in the absence of $CaCl_2$, and this delay was pronounced in the presence of $CaCl_2$. The delay in G1 to S transition in the Δ*ptp2* Δ*msg5* double disruptant was confirmed by monitoring over time the percentage of budded cells in cultures synchronized with -factor, a natural cell cycle inhibitor for G1 phase. After release from the α-factor arrest, the percentages of budded cells in the culture of the Δ*ptp2* Δ*msg5* double disruptant were three fold lower in the presence of 0.3 M $CaCl_2$ compared with those in the wild-type or in strains grown in the absence of calcium. DNA microarray analysis suggested that the G1-S transition delay observed in the ptp2 msg5 double disruptant in the presence of Ca^{2+} is caused by down-regulation of *CLN2*, further suggesting that activation of Cdc28p-G1 cyclin complex that is required for G1 to S progression was defective.

Chapter 3　Ptp2p and Msg5p are involved in cell integrity and vacuole morphogenesis

Since Ptp2p and Msg5p are known to be a negative regulator for the Slt2-cell wall integrity pathway, Western blot analysis was performed to investigate the involvement of the Slt2 pathway in the defective growth of the Δ*ptp2* Δ*msg5* double disruptant in the presence of high concentration of Ca^{2+}. Result revealed that Slt2p is hyper-activated, suggesting that the Ca^s-delay of G1 to S transition of the Δ*ptp2* Δ*msg5* double disruptant is related to hyper-activation of the Slt2 pathway.

Since vacuole serves as a storage organelle for excess calcium and its defect leads to sensitivity to Ca^{2+}, vacuole morphology of the Δ*ptp2* Δ*msg5* double disruptant was examined by staining the vacuolar membrane with FM4-64, a lypophilic styryl dye. Microscopic observation revealed that even in the absence of Ca^{2+} the vacuole of the Δ*ptp2* Δ*msg5* double disruptant was fragmented whereas that of either wild-type, Δ*ptp2* or Δ*msg5* was not, suggesting that Ptp2p and Msg5p are redundantly involved in vacuole morphogenesis.

Chapter 4　Identification of Δ*pkase* suppressors for the Ca^{2+} sensitivity of the Δ*ptp2* Δ*msg5* double disruptant

PKase genes implicated in the Ca^s-phenotype were identified by systematically constructing 101 triple disruptants having the genotype of Δ*ptp2*::Cg*HIS3* Δ*msg5*::Cg*LEU2* Δ*pkase*::kanMX4 (disruption of each of 101 non-essential PKase). It was discovered from this analysis that six non-essential PKase disruptions (Δ*bck1*, Δ*mkk1*, Δ*slt2*, Δ*mck1*, Δ*ssk2*, and Δ*yak1*) suppressed the Ca^s phenotype of the Δ*ptp2* Δ*msg5* double disruptant. Since suppression of the Ca^s-phenotype of the Δ*ptp2* Δ*msg5* double disruptant by Δ*pkase* disruption is not always accompanied by suppression of other phenotypes such as G1 to S delay, fragmented vacuole and Slt2 hyper-activation, these results suggest that at least two independent or parallel mechanisms contribute to the suppression of the Δ*ptp2* Δ*msg5* double disruptant.

Chapter 5　General discussion and conclusion

Based upon the observation in this study, it was proposed that the Ca^s-phenotype of the Δ*ptp2* Δ*msg5* double disruptant is caused by three different effects that could either reflect independent pathways or be part of a single cascade: i) insufficient activation of Cln2p-Cdc28p complex mediated by down-regulation of Cln2p resulting in a defect in G1 to S progression, ii) Ca^{2+}-mediated and Δ*ptp2* Δ*msg5* double disruption-mediated hyper-activation of Slt2p leading to a growth defect, and iii) fragmentation of vacuole disabling the Δ*ptp2* Δ*msg5* double disruptant to tolerate high concentrations of exogenous Ca^{2+}. How hyper-activation of Slt2 due to the absence of *PTP2* and *MSG5* is linked to down-regulation of *CLN2* and vacuole fragmentation awaits further analysis.

論文審査の結果の要旨

This dissertation presents an experimental work on functional redundancy of protein phosphatases, Ptp2p and Msg5p, for calcium stress response in budding yeast *Saccharomyces cerevisiae*. It was previously discovered by genetic screening that the Δ*ptp2* Δ*msg5* double disruptant displayed a calcium sensitive (Ca^s)-phenotype whereas the single disruptant of either *PTP2* or *MSG5* did not. This study was to identify and characterize pathways and molecular mechanism involved in Ca^s-phenotype of the Δ*ptp2* Δ*msg5* double disruptant. The main results obtained in this dissertation are summarized as follows:

In order to gain further insight into the molecular mechanism of Ca^s-phenotype of the Δ*ptp2* Δ*msg5* double disruptant, firstly it was investigated whether the Δ*ptp2* Δ*msg5* double disruptant exhibits growth arrest at any specific point of the cell cycle, particularly in the presence of exogenous Ca^{2+} by using FACS analysis. Result showed that the growth progression of the Δ*ptp2* Δ*msg5* double disruptant slowed down at the G1 to S transition even in the absence of $CaCl_2$, and this delay was pronounced in the presence of $CaCl_2$. The delay in G1 to S transition in the Δ*ptp2* Δ*msg5* double disruptant was confirmed by monitoring over time the percentage of budded cells in cultures synchronized with α-factor, a natural cell cycle inhibitor for G1 phase. DNA microarray analysis confirmed by RT PCR result suggested that the G1-S transition delay observed in the Δ*ptp2* Δ*msg5* double disruptant in the presence of Ca^{2+} is caused by down-regulation of *CLN2*.

Since Ptp2p and Msg5p are known to be a negative regulator for the Slt2-cell wall integrity pathway, the involvement of the Slt2 pathway in the defective growth of the Δ*ptp2* Δ*msg5* double disruptant in the presence of high concentration of Ca^{2+} was analyzed by Western blot analysis. Result revealed that hyper-activation of Slt2 pathway causes growth defect of the Δ*ptp2* Δ*msg5* double disruptant in the presence of Ca^{2+}. On the other hand, the vacuole of the Δ*ptp2* Δ*msg5* double disruptant was fragmented whereas that of either wild type, Δ*ptp2* or Δ*msg5* was not, suggesting that fragmented vacuoles correlates with Ca^s-phenotype of the Δ*ptp2* Δ*msg5* double disruptant.

The efforts to identify PKase genes implicated in the Ca^s-phenotype was carried out by systematically constructing 101 triple disruptants having the genotype of Δ*ptp2*::*CgHIS3* Δ*msg5*::*CgLEU2* Δ*pkase*::*kanMX4* (disruption of each of 101 non-essential PKase). From this analysis it was discovered that six non-essential PKase disruptions (Δ*bck1*, Δ*mkk1*, Δ*slt2*, Δ*mck1*, Δ*ssk2*, and Δ*yak1*) suppressed the Ca^s phenotype of the Δ*ptp2* Δ*msg5* double disruptant. Characterization of the Δ*ptp2* Δ*msg5* Δ*pkase* triple disruptant by FACS analysis, Western blot analysis of protein extracts and vacuole morphology observation suggested that at least two independent or parallel mechanisms, Slt2 and Hog pathways, contribute to the suppression of Ca^s phenotype of the Δ*ptp2* Δ*msg5* double disruptant

Based upon the data presented in this and other studies, Hermansyah proposes that the Ca^s-phenotype of the Δ*ptp2* Δ*msg5* double disruptant is caused by three different effects that could either reflect independent pathways or be part of a single cascade. i) Insufficient activation of Cln2-Cdc28p complex mediated by down-regulation of Cln2p results in a defect in G1 to S progression, ii) Ca^{2+}-mediated and Δ*ptp2* Δ*msg5* double disruption-mediated hyper-activation of Slt2p leads to a growth defect, and iii) fragmented vacuoles disable the Δ*ptp2* Δ*msg5* double disruptant to tolerate high concentrations of exogenous Ca^{2+}. These findings contributes to understanding the role of two protein phosphatases, Ptp2 and Msg5, in budding yeast as reference eukaryotes and provides a novel mechanism that two proteins phosphatases belonging to different subgroups work in redundant manner. Judging from these achievement, this dissertation deserves the degree of Doctor of Engineering.