



Title	Functional redundancy of protein phosphatases, Ptp2p and Msg5p, for calcium stress response in <i>Saccharomyces cerevisiae</i>
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Citation	大阪大学, 2010, 博士論文
Version Type	
URL	<a href="https://hdl.handle.net/11094/58313">https://hdl.handle.net/11094/58313</a>
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博士の専攻分野の名称	博 士 (工 学)
学 位 記 番 号	第 2 4 1 3 9 号
学 位 授 与 年 月 日	平 成 22 年 7 月 9 日
学 位 授 与 の 要 件	学位規則第4条第1項該当 工学研究科生命先端工学専攻
学 位 論 文 名	Functional redundancy of protein phosphatases, Ptp2p and Msg5p, for calcium stress response in <i>Saccharomyces cerevisiae</i> (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)
論 文 審 査 委 員	(主査) 教 授 原 島 俊  (副査) 教 授 藤 山 和 仁    教 授 渡 邊 肇    教 授 大 竹 久 夫 教 授 福 井 希 一    教 授 福 崎 英 一 郎    教 授 仁 平 卓 也 教 授 紀 ノ 岡 正 博    教 授 野 地 博 行    教 授 四 方 哲 也

## 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<sup>s</sup>) 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<sup>2+</sup> concentration induces delay of G1-S transition through down-regulation of *CLN2* expression in the $\Delta ptp2 \Delta msg5$ double disruptant

To investigate whether the  $\Delta ptp2 \Delta msg5$  double disruptant exhibits growth arrest at any specific point of the cell cycle, particularly in the presence of exogenous Ca<sup>2+</sup>, FACS analysis was conducted. It was found that growth progression of the  $\Delta ptp2 \Delta msg5$  double disruptant slowed down at the G1 to S transition even in the absence of CaCl<sub>2</sub>, and this delay was pronounced in the presence of CaCl<sub>2</sub>. The delay in G1 to

S transition in the  $\Delta ptp2 \Delta msg5$  double disruptant was confirmed by monitoring over time the percentage of budded cells in cultures synchronized with  $\alpha$ -factor, a natural cell cycle inhibitor for G1 phase. After release from the  $\alpha$ -factor arrest, the percentages of budded cells in the culture of the  $\Delta ptp2 \Delta msg5$  double disruptant were three fold lower in the presence of 0.3 M CaCl<sub>2</sub> 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<sup>2+</sup> 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 Slr2-cell wall integrity pathway, Western blot analysis was performed to investigate the involvement of the Slr2 pathway in the defective growth of the  $\Delta ptp2 \Delta msg5$  double disruptant in the presence of high concentration of Ca<sup>2+</sup>. Result revealed that Slr2p is hyper-activated, suggesting that the Ca<sup>s</sup>-delay of G1 to S transition of the  $\Delta ptp2 \Delta msg5$  double disruptant is related to hyper-activation of the Slr2 pathway.

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

### Chapter 4 Identification of $\Delta pkase$ suppressors for the Ca<sup>2+</sup> sensitivity of the $\Delta ptp2 \Delta msg5$ double disruptant

PKase genes implicated in the Ca<sup>s</sup>-phenotype were identified by systematically constructing 101 triple disruptants having the genotype of  $\Delta ptp2::CgHIS3 \Delta msg5::CgLEU2 \Delta pkase::kanMX4$  (disruption of each of 101 non-essential PKase). It was discovered from this analysis that six non-essential PKase disruptions ( $\Delta bck1$ ,  $\Delta mkk1$ ,  $\Delta slt2$ ,  $\Delta mck1$ ,  $\Delta ssk2$ , and  $\Delta yak1$ ) suppressed the Ca<sup>s</sup> phenotype of the  $\Delta ptp2 \Delta msg5$  double disruptant. Since suppression of the Ca<sup>s</sup>-phenotype of the  $\Delta ptp2 \Delta msg5$  double disruptant by  $\Delta pkase$  disruption is not always accompanied by suppression of other phenotypes such as G1 to S delay, fragmented vacuole and Slr2 hyper-activation, these results suggest that at least two independent or parallel mechanisms contribute to the suppression of the  $\Delta ptp2 \Delta msg5$  double disruptant.

### Chapter 5 General discussion and conclusion

Based upon the observation in this study, it was proposed that the Ca<sup>s</sup>-phenotype of the  $\Delta ptp2 \Delta msg5$  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<sup>2+</sup>-mediated and  $\Delta ptp2 \Delta msg5$  double disruption-mediated hyper-activation of Slr2p leading to a growth defect, and iii) fragmentation of vacuole disabling the  $\Delta ptp2 \Delta msg5$  double disruptant to tolerate high concentrations of exogenous Ca<sup>2+</sup>. How hyper-activation of Slr2 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  $\Delta ptp2 \Delta msg5$  double disruptant displayed a calcium sensitive ( $Ca^{2+}$ )-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  $Ca^{2+}$ -phenotype of the  $\Delta ptp2 \Delta msg5$  double disruptant. The main results obtained in this dissertation are summarized as follows:

In order to gain further insight into the molecular mechanism of  $Ca^{2+}$ -phenotype of the  $\Delta ptp2 \Delta msg5$  double disruptant, firstly it was investigated whether the  $\Delta ptp2 \Delta msg5$  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  $\Delta ptp2 \Delta msg5$  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  $\Delta ptp2 \Delta msg5$  double disruptant was confirmed by monitoring over time the percentage of budded cells in cultures synchronized with  $\alpha$ -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  $\Delta ptp2 \Delta msg5$  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  $\Delta ptp2 \Delta msg5$  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  $\Delta ptp2 \Delta msg5$  double disruptant in the presence of  $Ca^{2+}$ . On the other hand, the vacuole of the  $\Delta ptp2 \Delta msg5$  double disruptant was fragmented whereas that of either wild type,  $\Delta ptp2$  or  $\Delta msg5$  was not, suggesting that fragmented vacuoles correlates with  $Ca^{2+}$ -phenotype of the  $\Delta ptp2 \Delta msg5$  double disruptant.

The efforts to identify PKase genes implicated in the  $Ca^{2+}$ -phenotype was carried out by systematically constructing 101 triple disruptants having the genotype of  $\Delta ptp2::CgHIS3 \Delta msg5::CgLEU2 \Delta pkase::kanMX4$  (disruption of each of 101 non-essential PKase). From this analysis it was discovered that six non-essential PKase disruptions ( $\Delta bck1$ ,  $\Delta mkk1$ ,  $\Delta slt2$ ,  $\Delta mck1$ ,  $\Delta ssk2$ , and  $\Delta yak1$ ) suppressed the  $Ca^{2+}$  phenotype of the  $\Delta ptp2 \Delta msg5$  double disruptant. Characterization of the  $\Delta ptp2 \Delta msg5 \Delta pkase$  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  $Ca^{2+}$  phenotype of the  $\Delta ptp2 \Delta msg5$  double disruptant

Based upon the data presented in this and other studies, Hermansyah proposes that the  $Ca^{2+}$ -phenotype

of the  $\Delta ptp2 \Delta msg5$  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  $\Delta ptp2 \Delta msg5$  double disruption-mediated hyper-activation of Slt2p leads to a growth defect, and iii) fragmented vacuoles disable the  $\Delta ptp2 \Delta msg5$  double disruptant to tolerate high concentrations of exogenous  $Ca^{2+}$ . These findings contribute 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.