

Intracellular Ca²⁺ Regulation in Calcium Sensitive Phenotype of *Saccharomyces cerevisiae*

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Intracellular cytosolic Ca²⁺ concentration accumulation plays an essential information in *Saccharomyces cerevisiae* i.e. to explain cellular mechanism of Ca²⁺ sensitive phenotype. Disruption both *S. cerevisiae* PPase *PTP2* and *MSG5* genes showed an inhibited growth in the presence of Ca²⁺. On the other hand, by using Luminocounter with apoaequorin system, a method based upon luminescent photoprotein aequorin, intracellular Ca²⁺ concentration was accumulated as a consequence of calcium sensitive phenotype of *S. cerevisiae*. This fact indicated that PPase *ptp2Δ* and *msg5Δ* were involved in intracellular Ca²⁺ transport in addition their already known pathways i.e Mitogen Activated Protein Kinase cell wall integrity pathway, high osmolarity glycerol (HOG) pathway, and pheromone response *FUS3* pathway.

Key words: *Saccharomyces cerevisiae*, protein phosphatase, calcium sensitivity, calcium accumulation

INTRODUCTION

Ca²⁺ is used by virtually every eukaryotic cell to regulate a wide variety of cellular processes, including gene expression. A change in the cytosolic Ca²⁺ concentration plays a key regulatory role in diverse cellular processes, such as T-cell activation, muscle construction and neurotransmitter release (Clapham 1995). In the yeast *Saccharomyces cerevisiae*, Ca²⁺ has been involved in stress-induced expression on ion transporter genes, bud formation, viability upon pheromone-induced growth (Mizunuma *et al.* 2001).

Recently, we observed that in the presence of high Ca²⁺ concentration the growth of the *ptp2Δ msg5Δ* double disruptant was inhibited in G1 to S transition of cell cycle (Hermansyah *et al.* 2009) indicating that Ca²⁺ was involved in cellular mechanism of the *ptp2Δ msg5Δ* double disruptant. It can be that Ca²⁺ plays a key role in the transduction of external signal. Although the physiological role of Ca²⁺-mediated cell cycle regulation is poor understood, this regulation has been implicated in a mechanism that may be operate under conditions where cells encounter Ca²⁺-caused stress. Ca²⁺ is required at all stages of cell cycle except for the initiation of DNA synthesis (Shimada *et al.* 1991). Fast adaptation to stressing environmental changes is often requirement for cell survival, including either post transcriptional mechanism, adaptation involves remodeling gene expression and is mediated by induction or repression of a more or less specific set of genes (Estruch 2000).

PTP2 gene encodes protein tyrosine phosphatase which involved in Hog1p mitogen activated protein (MAP) kinase high-osmolarity sensing pathway and Fus3p MAP kinase pheromone, while *MSG5* is dual specificity protein

tyrosine phosphatase which involved in adaptation response to pheromone (Watanabe *et al.* 1995; Jacoby *et al.* 1997; Mattison *et al.* 1999; Zhan & Guan 1999; Flandez *et al.* 2004).

In this study *S. cerevisiae* intracellular Ca²⁺ concentration was determined based upon its reaction with aequorin and detected using Luminocounter. Aequorin has a high specificity for Ca²⁺, therefore it can be used as a biological indicator of Ca²⁺. Aequorin is a photoprotein containing coelenterazine as a chromophore originally found in the jellyfish *Aequorea victoria*. The binding of Ca²⁺ to aequorin generates transient bioluminescence, yielding as light products, CO₂, and a blue fluorescent protein.

Disruption of *PTP2* and *MSG5* which leads to calcium sensitive phenotype caused a accumulation of intracellular Ca²⁺ concentration, indicating that Ca²⁺ transport might implicate in the cellular mechanism of calcium sensitive phenotype of the *ptp2Δ msg5Δ* double disruptant *S. cerevisiae*.

MATERIALS AND METHODS

Yeast Strains, Plasmids, and Culture Conditions.

Yeast strains BY5209 (=FY833) *MATα.ura3-52 his3-Δ200 leu2Δ1 lys2Δ202 trp1Δ63* and BY5210 (=FY834) *MATα.ura3-52 his3-Δ200 leu2Δ1 lys2Δ202 trp1Δ63* (Winston *et al.* 1995) were used as a wild-type and parental strains, and the *ptp2Δ msg5Δ* double disruptant *MATα.ura3-52 his3-Δ200 leu2Δ1 lys2Δ202 trp1Δ63*. The rich medium YPAD was prepared by supplementing YPD broth (Sigma-Aldrich Co.) with 0.4 mg/ml adenine. SC medium consisted of 0.67% yeast nitrogen base without amino acids, 2% glucose and

the required auxotrophic supplements. SPM medium contained 0.30% potassium acetate, 0.02% raffinose and was supplemented with 10 µg/ml of adenine, arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, uracil, and valine. Unless indicated otherwise, yeast strains were grown at 30 °C. Plasmids were propagated in *Escherichia coli* strain DH5 α cultivated on LB medium containing 100 µg/ml ampicillin at 37 °C. Synthetic medium (SD) contained 0.67% Bacto yeast nitrogen base without amino acids and 2% Bacto-peptone, and 2% glucose. In calcium deficient medium (SD-Ca), CaCl₂ was omitted and calcium pantothenate was replaced by sodium pantothenate (Shimada *et al.* 1991). The concentration of calcium in SD-Ca medium was 0.24 µM. The *ptp2Δ msg5Δ* double disruptant was constructed by crossing between *ptp2Δ::CgHIS3* single disruptant and *msg5Δ::CgLEU2* single disruptant (Hermansyah *et al.* 2009). The expression plasmid pGAPAQ1 was constructed by inserting the *SacII/EcoRI* 0.6-kilobase (kb) fragment of the apoaequorin cDNA into the pYSI vector at the *EcoRI* site.

Introducing Plasmid pGAPAQ1 Using Yeast Transformation with Li/PEG/SS Methods. Yeast cells were inoculated on YPDA or appropriate media, the cultivation was refreshed in 5 ml of YPDA or appropriate medium. It was added 0.5 ml of culture to 5 ml of fresh medium and cultivate for 3-4 hours to reach mid mid-log phase ($OD_{600} = 1.0$). The culture was harvested in a sterile centrifuge tube at 2,000 rpm for 5 minutes. Then the medium was poured off, and washed with 5 ml of sterile water, subsequently centrifuge again. After discarded the water, cells were resuspended in 1 ml of 0.1 M LiAc and transferred to a sterile 1.5 ml tube. Pellet of cells were obtained after spinned at 12,000 rpm for 30 sec and LiAc was removed. LiAc (0.5 ml of 0.1 M) was added and store at 30 °C. Single-strand carrier DNA was boiled for 5 min and quickly chilled in ice for 5 min. After vortex the cell suspension, 0.1 ml of sample (increase volume if cells are less dense) was pipetted into a 1.5 ml tube, and cells pellet were obtained by spinned at 10,000 xg for 30 sec, and then LiAc was remove. Then the following reagents were added carefully in the order listed; 0.24 ml of 50% (w/v) PEG 4,000, 0.036 ml of 1.0 M LiAc (overlay carefully on 50% PEG), 0.005 ml of carrier DNA (10 mg/ml), 0.070 ml of plasmid DNA (0.1-10 µg) and sterile water. Pellet cell was vortex vigorously until completely mixed (approximately 1 min) and was incubated for 30 min at 30 °C prior to heat shocked for 20-25 min at 42 °C. Pellet was centrifuged at 10,000 xg for 1 min, the liquid was removed and sterile water was added (0.1 ml). Then the pellet resuspended by pipetting gently, and plate onto selective a plate. Single colonies will appear after 2-3 days incubation at 30 °C. Plasmid DNA is a 400 µl PCR product which precipitated by a mixture of 1/10 volume 3 M NaCH₃COO pH 5.2 and 2.5 volume ethanol, was centrifuged in 10,000 xg 4°C for 1 min, subsequently dissolved in 70 µl a sterile water.

Yeast transformant *ptp2Δ msg5Δ* containing either plasmid pGAPAQ1 or pYSI was selected in selective medium SC medium without his-, leu- and trp-, while wild

type containing either pGAPAQ1 or pYSI was selected in selective media SC medium without trp-. Each candidate disruptants were transferred into new appropriate media in order to obtain stable transformant.

Measurement of Intracellular Calcium by Luminocounter Using Apoaequorin Protein. Cells were harvested when they reached 5.10⁶ cells/ml and cells were counted by using hemicytometer. After centrifuge at 3,000 rpm, 30 °C, 5 min. the supernatant was discharged using aspirator. A total of 299 µl SD medium without 1 M sorbitol was prepared and 1 µl of 1.5 mM coelenterazine was added and the solution was mixed using sonicator. This solution was added into cells prior to incubated cells solution in water bath incubator using black cover at 25 °C, 20 min. The cells were filtered using Millipore and aspirator. The cells were washed by one and then by nine ml SD medium, respectively, and cells were resuspended with 2 x 250 µl SD medium.

Determination of Intracellular Ca²⁺ Concentration Using Luminocounter. Procedure for intracellular Ca²⁺ concentration using Luminocounter was as follows: (i) cells sample were placed into sample cup (time 32 min), (ii) measurement was started (time 34 min), (iii) hypoosmotic stress was injected 600 µl H₂O (time 35 min), (iv) 4% Triton X-100/4M CaCl₂ was injected (time 39 min), and (v) measurement was finished at time 40 min.

RESULTS

Transformation of Wild Type and the *ptp2Δ msg5Δ* Double Disruptant Used pGAPAQ1 Bearing Apoaequorin Gene. The plasmid expression pGAPAQ1 was constructed by inserting the *SacII/EcoRI* 0.6 kilobase the apoaequorin cDNA fragment into the pYSI vector at the *EcoRI* site (Figure 1) (Shimada *et al.* 1991). Plasmid pYSI was a cloning vector based on a YE_p (yeast episomal vector) and contained a *S. cerevisiae* glyceraldehydes-3-phosphate dehydrogenase (GAPD) as a promoter and *TRP1* as a selection marker, carried 2µplasmid DNA sequence necessary for autonomous replication. The GAPD

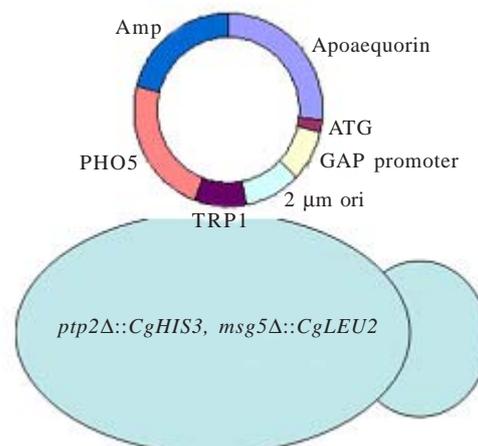


Figure 1. Plasmid pGAPAQ1 which bears Apoaequorin cDNA for measurement of intracellular by luminescent photoprotein aequorin.

promoter by which the cDNA is expressed constitutively was used because yeast cell growth is not effected by conditional expression of apoaequorin under the control of the *GAL1* promoter. Before introducing the plasmid pGAPAQ1 and pYS1 into wild type and *ptp2Δ msg5Δ*, those plasmids (pGAPAQ1 and pYS1) was introduced into *E. coli* DH5 α (Figure 2).

Plasmid pYS1 size was 10.7 kb, restricted by *Bam*HI restriction enzyme generated one fragment 10.7 kb. While size of plasmid pGAPAQ1 10.7 kb + 0.63 kb = 11.33 kb, and restricted by *Bam*HI generated two fragment, 9.9 kb and less than 1.4 kb (Figure 3).

Measurement of Intracellular Ca²⁺ Concentration Using Luminocounter. Intracellular Ca²⁺ concentration was measured with an apoaequorin cDNA system by using Luminocounter. The result showed that in SC-trp containing Ca²⁺ double disruption of *PTP2* and *MSG5* but not in wild type due to intracellular Ca²⁺ accumulation. On the other hand, in the SC-trp without Ca²⁺ culture either the *ptp2Δ msg5Δ* double disruptant or wild type did not cause intracellular Ca²⁺ accumulation (Figure 4).

DISCUSSION

Limitations in the direct measurement of intracellular Ca²⁺ and difficulties in quantifying Ca²⁺ channel activity in yeast are a problem in progress to understand Ca²⁺ signaling. By using Luminocounter with apoaequorin system, a method based upon luminescent photoprotein aequorin, intracellular Ca²⁺ concentration was changed as a consequence of calcium sensitive phenotype of *S.*

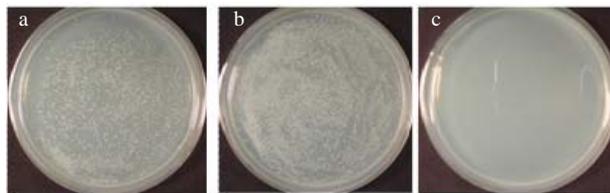


Figure 2. Transformation of DH5 α with (a) plasmid pGAPAQ1; (b) plasmid pYS1; (c) Negative control.

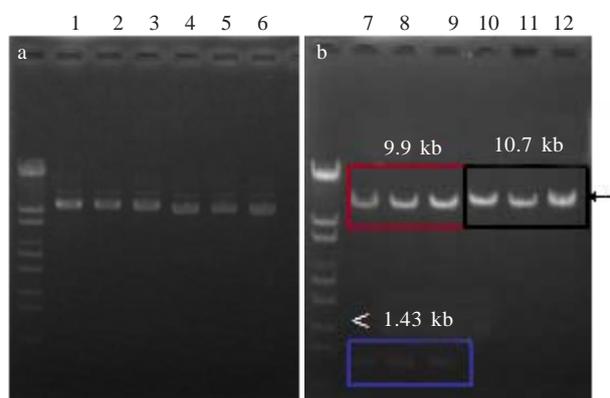


Figure 3. Agarose gel electrophoresis analysis of pGAPAQ1 and pYS1. (a) undigested (b) Digested using *Bam*HI restriction enzyme. Line 1, 2, 3, 7, 8, 9 is pGAPAQ1; Line 4, 5, 6, 10, 11, and 12 is pYS1. The Marker used λ /EcoT14.

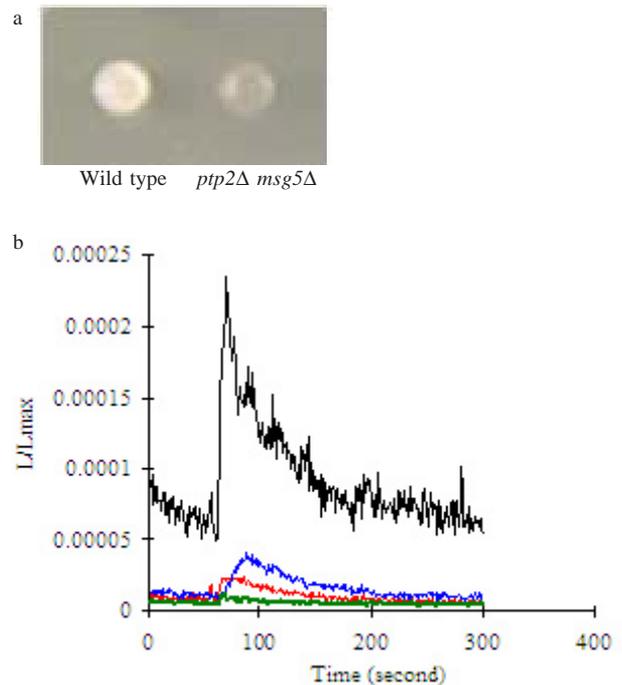


Figure 4. Measurement of intracellular Ca²⁺ with an apoaequorin system by using Luminocounter. Correlation between Ca²⁺ sensitive phenotype with intracellular Ca²⁺ concentration. (a) *ptp2Δ msg5Δ* Ca²⁺ showed sensitive phenotype. (b) In SC-trp containing Ca²⁺ the *ptp2Δ msg5Δ* double disruptant but not in wild type showed accumulation of intracellular Ca²⁺. While in the SC-trp without Ca²⁺ culture either the *ptp2Δ msg5Δ* double disruptant or wild type did not cause intracellular Ca²⁺ accumulation. —: WT apo, —: WT apo Ca, —: ptp2m sg5 apo, —: ptp2m sg5 apo Ca.

cerevisiae. The result suggests that both *PTP2* and *MSG5* are implicated in regulation of *S. cerevisiae* intracellular Ca²⁺. Transient increases in intracellular Ca²⁺ regulate a wide variety of cellular processes and Ca²⁺ signaling. Overlapping Ca²⁺ sensitive phenotype of the *ptp2Δ msg5Δ* double disruptant with its accumulation of intracellular Ca²⁺ concentration indicates that it has relationship between those two phenomenon. It was reported that lacking of Pmr1p, a golgi-localized Ca²⁺ transporting ATPase showed growth sensitivity to Ca²⁺ (Yadav *et al.* 2007). One need to elucidate more detail whether both PPase Ptp2p and Msg5p also involved in Ca²⁺ transport in cellular.

Aequorin is a reaction complex of apoaequorin, coelenterazine as a substrate, and molecular oxygen. This reaction results bioluminescence triggered by ion Ca²⁺ by following mechanism; the coelenterazine is oxidized to coelenteramide, and then the excited state of coelenteramide bound to apoaequorin (blue fluorescent protein) emit in the reaction. While In vitro Aequorin is regenerated by incubation with coelenterazine, molecular oxygen, 2-mercaptoethanol, pantothenate (Iida *et al.* 1990).

Accumulation of cytosolic Ca²⁺ could be occurred when two Ca²⁺ pumps, *PMC1* and *PMR1* which functions maintaining cytosolic free Ca²⁺ at submicromolar levels in budding yeast were deleted, and this cytosolic Ca²⁺ accumulation causes lethal (Cunningham & Fink 1994).

Further, yeast cells lacking of Pmr1p, Ca²⁺-ATPase pump are unable to maintenance proper level of Ca²⁺ within golgi apparatus because increase in Ca²⁺ uptake rate (Kellermeyer *et al.* 2003).

It is previously reported that vacuole morphology of the *ptp2 msg5* double disruptant by staining the vacuolar membrane with FM4-64, a lipophilic styryl dye even in the absence of Ca²⁺ the vacuole of the $\Delta ptp2 \Delta msg5$ double disruptant was fragmented (Hermansyah *et al.* 2009). This fragmented vacuolar correlate with increase in intracellular cytosolic Ca²⁺ (Kellermeyer *et al.* 2003). Thus, *PTP2* and *MSG5* may function or involved in intracellular Ca²⁺ transport with redundant manner because *S. cerevisiae* yeast strain lacking both Ptp2p and Msg5p showed Ca²⁺ sensitive growth, intracellular Ca²⁺ concentration accumulation and vacuole fragmentation.

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