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Intracellular Ca^{2+} Regulation in Calcium Sensitive Phenotype of *Saccharomyces cerevisiae*

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Intracellular cytosolic Ca^{2+} concentration accumulation plays an essential information in *Saccharomyces cerevisiae* i.e. to explain cellular mechanism of Ca^{2+} sensitive phenotype. Disruption both *S. cerevisiae* PPase *PTP2* and *MSG5* genes showed an inhibited growth in the presence of Ca^{2+}. On the other hand, by using Luminocounter with apoaequorin system, a method based upon luminescent photoprotein aequorin, intracellular Ca^{2+} concentration was accumulated as a consequence of calcium sensitive phenotype of *S. cerevisiae*. This fact indicated that PPase *ptp2*Δ and *msg5*Δ were involved in intracellular Ca^{2+} 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

Ca^{2+} is used by virtually every eukaryotic cell to regulate a wide variety of cellular processes, including gene expression. A change in the cytosolic Ca^{2+} 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*, Ca^{2+} 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 Ca^{2+} concentration the growth of the *ptp2*Δ *msg5*Δ double disruptant was inhibited in G1 to S transition of cell cycle (Hermansyah *et al.* 2009) indicating that Ca^{2+} was involved in cellular mechanism of the *ptp2*Δ *msg5*Δ double disruptant. It can be that Ca^{2+} plays a key role in the transduction of external signal. Although the physiological role of Ca^{2+}-mediated cell cycle regulation is poor understood, this regulation has been implicated in a mechanism that may be operate under conditions where cells encounter Ca^{2+}-caused stress. Ca^{2+} 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 respone 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 Ca^{2+} concentration was determined based upon its reaction with aequorin and detected using Luminocounter. Aequorin has a high specificity for Ca^{2+}, therefore it can be used as a biological indicator of Ca^{2+}. Aequorin is a photoprotein containing coelenterazine as a chromophore originally found in the jellyfish *Aequorea victoria*. The binding of Ca^{2+} to aequorin generates transient bioluminescence, yielding as light products, CO_2, and a blue fluorescent protein.

Disruption of *PTP2* and *MSG5* which leads to calcium sensitive phenotype caused a accumulation of intracellular Ca^{2+} concentration, indicating that Ca^{2+} transport might implicate in the cellular mechanism of calcium sensitive phenotype of the *ptp2*Δ *msg5*Δ double disruptant *S. cerevisiae*.

MATERIALS AND METHODS

Yeast Strains, Plasmids, and Culture Conditions. Yeast strains BY5209 (= FY833) *MAT*α *ura3-52 his3-*Ä*200 leu2*Ä*1 lys2*Ä*202 trp1*Ä*63*and BY5210 (=FY834) *MAT ura3-52 his3-*Ä*200 leu2*Ä*1 lys2*Ä*202 trp1*Ä*63* (Winston *et al*. 1995) were used as a wild-type and parental strains, and the *ptp2*Δ *msg5*Δ double disruptant *MAT*α *ptp2*Δ::*CgHIS3 msg5*::*CgLEU2 ura3-52 his3-*Ä*200 leu2*Ä*1 lys2*Ä*202 trp1*Ä*63*. 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 DH5α 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), $CaCl_2$ 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 *ptp2*Δ *msg5*Δ double disruptant was constructed by crossing between *ptp2*Δ::*CgHIS3* single disruptant and *msg5*Δ::*CgLEU2* single disruptant (Hermansyah *et al*. 2009). The expression plasmid pGAPAQ1 was constructed by inserting the *Sac*II/*Eco*RI 0.6-kilobase (kb) fragment of the apoaequorin cDNA into the pYSI vector at the *Eco*RI 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 transfered 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 $NaCH_3COO$ 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 *ptp2*Δ *msg5*Δ 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 appreciate media in order to obtain stable transformant.

Measurement of Intracellular Calcium by Luminocounter Using Apoaequorin Protein. Cells were harvested when they reached 5.10^6 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 Ca^{2+} Concentration Using Luminocounter. Procedure for intracellular Ca^{2+} 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 H_2O (time 35 min), (iv) 4% Triton X-100/4M $CaCl_2$ 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 *Sac*II/*Eco*RI 0.6 kilobase the apoaequorin cDNA fragment into the pYSI vector at the *Eco*RI site (Figure 1) (Shimada *et al*. 1991). Plasmid pYSI was a cloning vector based on a YEp (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

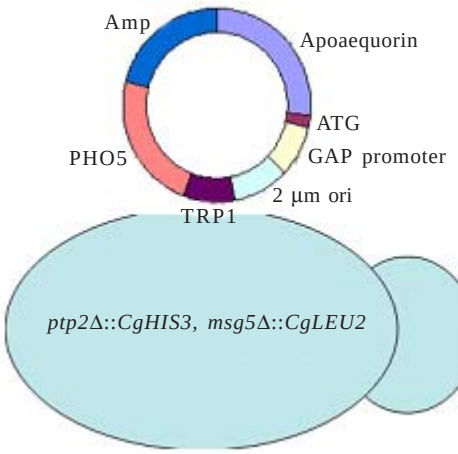

Figure 1. Plasmid pGAPAQ1 which bears Apoaequorin cDNA for measurement of intracellular by luminescent photoprotein aequorin.

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 *ptp2*Δ *msg5*Δ, those plasmids (pGAPAQ1 and pYS1) was introduced into *E. coli* DH5α (Figure 2).

Plasmid pYS1 size was 10.7 kb, restricted by *BamH1* 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 *BamH1* generated two fragment, 9.9 kb and less than 1.4 kb (Figure 3).

Measurement of Intracellular Ca^{2+} Concentration Using Luminocounter. Intracellular Ca^{2+} concentration was measured with an apoaequorin cDNA system by using Luminocounter. The result showed that in SC-trp containing Ca^{2+} double disruption of *PTP2* and *MSG5* but not in wild type due to intracellular Ca^{2+} accumulation. On the other hand, in the SC-trp without Ca^{2+} culture either the *ptp2*Δ *msg5*Δ double disruptant or wild type did not cause intracellular Ca^{2+} accumulation (Figure 4).

DISCUSSION

Limitations in the direct measurement of intracellular Ca^{2+} and difficulties in quantifying Ca^{2+} channel activity in yeast are a problem in progress to understand Ca^{2+} signaling. By using Luminocounter with apoaequorin system, a method based upon luminescent photoprotein aequorin, intracellular Ca^{2+} concentration was changed as a consequence of calcium sensitive phenotype of *S. cerevisiae*. The result suggests that both *PTP2* and *MSG5* are implicated in regulation of *S. cerevisiae* intracellular Ca^{2+}. Transient increases in intracellular Ca^{2+} regulate a wide variety of cellular processes and Ca^{2+} signaling. Overlapping Ca^{2+} sensitive phenotype of the *ptp2*Δ *msg5*Δ double disruptant with its accumulation of intracellular Ca^{2+} concentration indicates that it has relationship between those two phenomenon. It was reported that lacking of Pmr1p, a golgi-localized Ca^{2+} transporting ATPase showed growth sensitivity to Ca^{2+} (Yadav *et al*. 2007). One need to elucidate more detail whether both PPase Ptp2p and Msg5p also involved in Ca^{2+} transport in cellular.

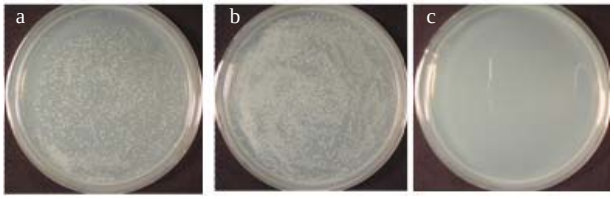

Figure 2. Transformation of DH5α with (a) plasmid pGAPAQ1; (b) plasmid pYS1; (c) Negative control.

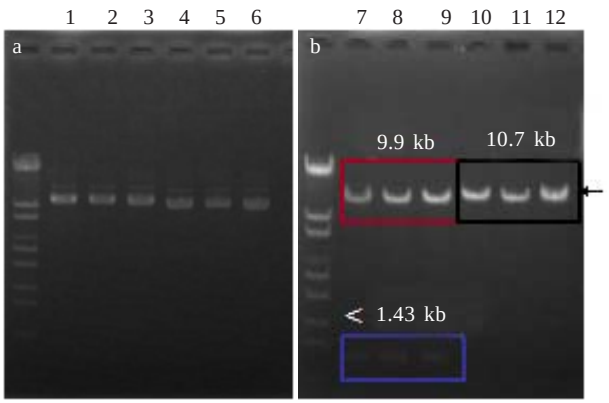

Figure 3. Agarose gel eletrophoresis analysis of pGAPAQ1 and pYS1. (a) undigested (b) Digested using *BamHI* restriction enzyme. Line 1, 2, 3, 7, 8, 9 is pGAPAQ1; Line 4, 5, 6, 10, 11, and 12 is pYS1. The Marker used λ/EcoT14.

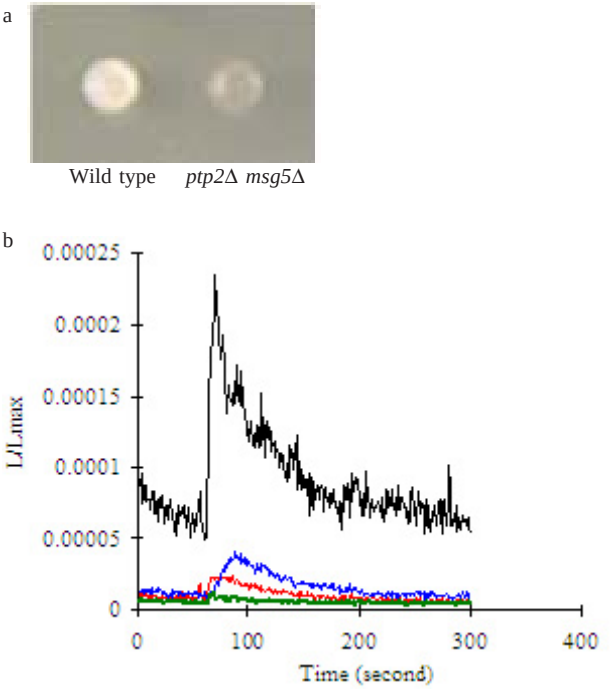

Figure 4. Measurement of intracellular Ca^{2+} with an apoaequorin system by using Luminocounter. Correlation between Ca^{2+} sensitive phenotype with intracellular Ca^{2+} concentration. (a) *ptp2*Δ *msg5*Δ Ca^{2+} showed sensitive phenotype. (b) In SC-trp containing Ca^{2+} the *ptp2*Δ *msg5*Δ double disruptant but not in wild type showed accumulation of intracellular Ca^{2+}. While in the SC-trp without Ca^{2+} culture either the *ptp2*Δ *msg5*Δ double disruptant or wild type did not cause intracellular Ca^{2+} accumulation. ——: WT apo, ——: WT apo Ca, ——: ptp2m sg5 apo, ——: ptp2m sg5 apo Ca.

Aequorin is a reaction complex of apoaequorin, coelenterazine as a substrate, and molecular oxygen. This reaction results bioluminescence triggered by ion Ca^{2+} 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 Ca^{2+} could be occurred when two Ca^{2+} pumps, *PMC1* and *PMR1* which functions maintaining cytosolic free Ca^{2+} at submicromolar levels in budding yeast were deleted, and this cytosolic Ca^{2+} accumulation causes lethal (Cunningham & Fink 1994).

Further, yeast cells lacking of Pmr1p, Ca^{2+}-ATPase pump are unable to maintenance proper level of Ca^{2+} within golgi apparatus because increase in Ca^{2+} 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 lypophilic styryl dye even in the absence of Ca^{2+} the vacuole of the Δ*ptp2* Δ*msg5* double disruptant was fragmented (Hermansyah *et al*. 2009). This fragmented vacuolar correlate with increase in intracellular cytosolic Ca^{2+} (Kellermeyer *et al*. 2003). Thus, *PTP2* and *MSG5* may function or involved in intracellular Ca^{2+} transport with redundant manner because *S. cerevisiae* yeast strain lacking both Ptp2p and Msg5p showed Ca^{2+} sensitive growth, intracellular Ca^{2+} concentration accumulation and vacuole fragmentation.

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