The Journal of Microbiology, February 2008, p. 70-74
DOI 10.1007/s12275-007-0244-y
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Vol. 46, No. 1

Nitrogen Depletion Causes Up-Regulation of Glutathione Content and γ-Glutamyltranspeptidase in *Schizosaccharomyces pombe*

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(Received December 11, 2007 / Accepted January 10, 2008)

This work aims to elucidate the relationship between nitrogen depletion and Glutathione (GSH) level in *Schizosaccharomyces pombe*. The total GSH level was much higher in the Pap1-positive KP1 cells than in the Pap1-negative TP108-3C cells, suggesting that synthesis of GSH is dependent on Pap1. When the Pap1-positive KP1 cells were transferred to the nitrogen-depleted medium, total GSH level significantly increased up to 6 h and then slightly declined after 9 h. Elevation of the total GSH level was observed to be much less with the Pap1-negative cells. However, glucose deprivation was not able to enhance the GSH level in the KP1 cells. Activity of γ-glutamyltranspeptidase (γ-GT), an enzyme in the first step of GSH catabolism, also increased during nitrogen depletion. The total GSH level was more significantly enhanced in the KP1 cells overexpressing γ-GT2 than γ-GT1 during nitrogen starvation. Reactive oxygen species (ROS) levels were not changed during nitrogen starvation in both Pap1-positive and Pap1-negative cells. Collectively, nitrogen depletion causes up-regulation of GSH synthesis and γ-GT in a Pap1-dependent manner.

***Keywords*:** fission yeast, glutathione, γ-glutamyltranspeptidase, Pap1, *Schizosaccharomyces pombe*

Microbial cells, including budding and fission yeasts, frequently encounter nutritional limitations in nature, and adapt themselves to the adverse conditions by controlling the expression of various genes. For the *Saccharomyces cerevisiae* cells starved for carbon, carbon and nitrogen, or nitrogen in the presence of glucose, the metabolic capacity is controlled by the glucose transport ability with some influence from several glycolytic enzymes (Albers *et al.*, 2007). This is partly supported by another finding which shows that nitrogen starvation results in the lack of tolerance due to degradation of the glucose uptake system in cells starved for nitrogen in the presence of a fermentable carbon source (Busturia and Lagunas, 1986). As nitrogen starvation progresses, a general downregulation of genes associated with catabolism is observed. On the contrary, expression of genes encoding ribosomal proteins and involved in ribosome biogenesis was slightly increased during nitrogen starvation (Mendes-Ferreira *et al.*, 2007).

In the fission yeast *Schizosaccharomyces pombe*, haploid cells are completely arrested under nitrogen starvation with high viability (Shimanuki *et al.*, 2007). Autophage, a conserved bulk protein degradation process, occurs in the fission yeast functions to supply nitrogen and is activated when cells cannot access exogenous nitrogen, thus ensuring that they can adapt and subsequently propagate (Kohda *et al.*, 2007). *S. pombe* Isp6(+), a vascuolar serine protease specifically induced during nitrogen starvation, contributes to sexual development by providing a nitrogen source through autophagy (Nakashima *et al.*, 2006). Tor2-depleted fission yeast cells exhibit a phenotype very similar to that of wild-type cells starved for nitrogen, including arrest at the G1 phase of the cell cycle, induction of nitrogen-starvation-specific genes, and entrance into the sexual development pathway, which is in contrast to the failure of tor1 mutants to initiate sexual development or arrest in G1 under nitrogen starvation (Matsuo *et al.*, 2007; Weisman *et al.*, 2007). The fission yeast Tip41, a regulator of TAP42 required for Tor regulation, also plays a partial role in cellular responses to nitrogen nutrient conditions through regulation of type 2A phosphatase activity (Fenyvuesvolgyi *et al.*, 2005). The tip41 mutant cells also enter the G1 phase earlier than wild-type cells in response to nitrogen starvation, while overexpression of tip41(+) causes cell death, spherical cell morphology and blocks the shift to the G1 phase upon nitrogen starvation (Fenyvuesvolgyi *et al.*, 2005). In this study, we demonstrate that the total GSH level and γ-glutamyltranspeptidase activity are up-regulated in the nitrogen-starved *S. pombe* cells.

Materials and Methods

Chemicals

Bovine serum albumin (BSA), L-leucine, uracil, adenine, glucose, glutathione (GSH), glutathione reductase, Bradford reagent, 5,5'-dithiobis-(2-nitrobenzoic acid), L-γ-glutamyl-*p*-nitroanilide, glycine-glycine, and NADPH were purchased from Sigma Chemical Co. (USA). Yeast extract and agar were obtained from Amersham Life Science (USA). 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) was obtained from Invitrogen (USA). All other chemicals used were of highest grade commercially available.

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Strains and growth conditions

S. pombe KP1 (*h^+ leu1-32 ura4-294*) and TP108-3C (*h^- leu1 ura4 pap1::$ura4^+$*) were typically used. The yeast cells were grown in minimal medium, which contains KH phthalate (3 g), Na_2HPO_4 (1.8 g), NH_4Cl (5 g), D-glucose (20 g), 1,000× vitamin mixture (1 ml), 10,000× minerals (0.1 ml), 50× salts (20 ml), and L-leucine (250 mg) per 1 L. Salts stock (50×) contains 5.2 mM $MgCl_2{\cdot}6H_2O$, 0.1 mM $CaCl_2{\cdot}2H_2O$, 13.4 mM KCl, and 0.28 mM Na_2SO_4. Minerals stock (10,000×) contains 8.1 μM H_3BO_3, 2.37 μM $MnSO_4$, 1.39 μM $ZnSO_4{\cdot}7H_2O$, 0.74 μM $FeCl_3{\cdot}6H_2O$, 0.25 μM $MoO_4{\cdot}2H_2O$, 0.6 μM KI, 0.16 μM $CuSO_4{\cdot}5H_2O$, and 4.76 μM citric acid. Vitamins stock (1,000×) contains 81.2 μM nicotinic acid, 55.5 μM inositol, 40.8 μM biotin, and 4.2 μM pantothenic acid. The yeast cells were shaken at 30°C, and the cell growth was monitored by culture absorbance at 600 nm.

Plasmids

The recombinant plasmids pPHJ98 (Park *et al.*, 2004) and pPHJ02 (Park *et al.*, 2005), which contain the full-length genomic DNA of γ-GT1 and γ-GT2 from *S. pombe*, respectively, were used. The *E. coli*-yeast shuttle vector pRS316 (Myers *et al.*, 1986) was used for the vector control cells.

Cell harvest and preparation of cellular extracts

The appropriate number of the yeast cells was harvested by centrifugation. They were resuspended in 20 mM Tris buffer (pH 8.0)-2 mM EDTA and disrupted using a glass bead beater. Supernatant was obtained after centrifugation and used as crude extract for GSH determination, enzyme assay and protein determination.

Determination of total GSH

Total GSH in extracts was determined as previously described by Floreani *et al.* (1997). However, the reaction mixture (200 μl) contained 160 μl of phosphate buffer (pH 7.4)-10 mM EDTA, 10 μl of 4.8 mM NADPH, 5 μl of 4 mM 5,5'-ithiobis-2-nitrobenzoic acid) and 20 μl of cellular extract. After 3 min of equilibration at 25°C, the reaction was started by adding 5 μl of 6 units/ml glutathione reductase, and the formation of 2-nitro-5-thiobenzoic acid was recorded at 412 nm.

Enzyme assay and protein determination

γ-Glutamyltranspeptidase (γ-GT) activity in yeast extracts was spectrophotometrically determined with L-γ-glutamyl-*p*-nitroanilide as a donor and glycyl-glycine as an acceptor (Sulakhe, 1986). The assay mixture contained 4.6 mM L-γ-glutamyl-*p*-nitroanilide, 100 mM glycyl-glycine, 100 mM Tris-Cl; pH 8.0, and 10~60 μg protein in a final volume of 200 μl. The enzymatically liberated *p*-nitroaniline was detected at 410 nm. Protein content in extracts was determined according to the procedure of Bradford using BSA as a standard (Bradford, 1976).

Determination of intracellular reactive oxygen species

For analysis of intracellular reactive oxygen species (ROS), the redox-sensitive fluorescent probe DCFH-DA as used, as previously described by Royall and Ischiropoulos (1993). The yeast cells were incubated with 5 μM DCFH-DA for 30 min at 37°C. The harvested cells were immediately analyzed by flow cytometry. DCFH-DA is non-fluorescent and can readily diffuse across cell membranes and hydrolyzed by intracellular esterases to non-fluorescent 2',7'-dihydrodichlorofluorescein (DCFH), which is converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF) after reacting with intracellular ROS.

Results and Discussion

Basal synthesis of GSH dependent on Pap1

GSH, an important antioxidant involved in stress response, is synthesized in two sequential reactions involving γ-glutamylcysteine synthetase, followed by glutathione synthetase. In previous studies, the two synthetic enzymes in *S. pombe* were identified to be regulated by oxidative, nitrosative or nutritional stress (Kim *et al.*, 2004a, 2004b, 2004c). The transcription factor Pap1 is involved in the induction of the γ-glutamylcysteine synthetase gene by superoxide radicals, whereas induction of the γ-glutamylcysteine synthetase gene occurring due to low glucose concentration does not depend on the presence of Pap1 (Kim *et al.*, 2004a). However, expression of the glutathione synthetase gene is induced by low glucose, fructose and disaccharides, dependent on Pap1 (Kim *et al.*, 2004b). These findings suggest that the two GSH synthetic enzymes are not always subject to the same control mechanisms. To examine whether Pap1 is involved in maintaining basal level of GSH, the total GSH levels were detected in Pap1-positive *S. pombe* KP1 and Pap1-negative *S. pombe* TP108-3C cells. As shown in Fig. 1, the GSH content in the KP1 cells was 3-fold higher than that in the TP108-3C cells. In brief, the basal synthesis of GSH in *S. pombe* is dependent on Pap1.

Nitrogen starvation causes up-regulation of GSH content

The status of nutrients are the primary control drivers of growth and development in yeasts. When *S. pombe* cells are

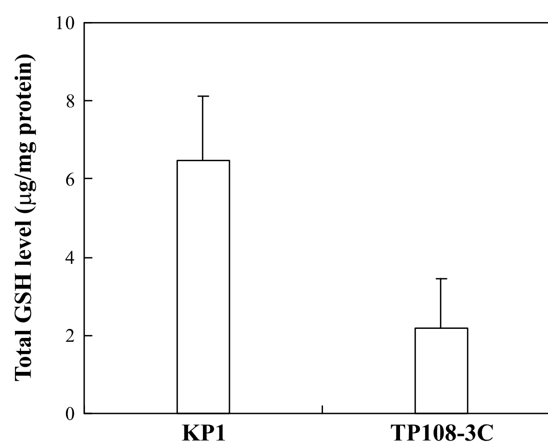

Fig. 1. Total GSH contents in Pap1-positive *S. pombe* KP1 and Pap1-negative *S. pombe* TP108-3C cells under exponential growth. The two yeast cultures, exponentially grown, were transferred to NH_4Cl-deficient media for 6 h. The GSH content in extracts was spectrophotometrically determined as described in 'Materials and Methods'.

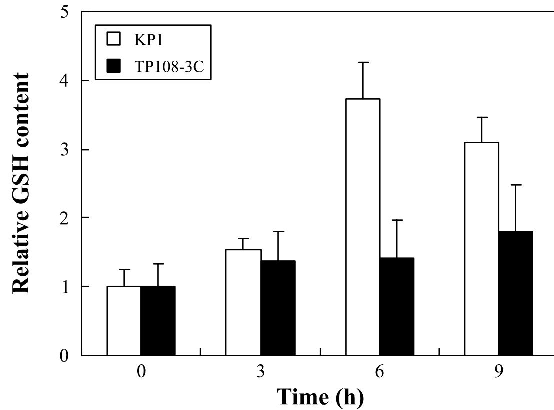

Fig. 2. Variations in total GSH contents of Pap1-positive *S. pombe* KP1 and Pap1-negative *S. pombe* TP108-3C cells during nitrogen starvation. Each column represents Mean±SE The yeast cultures were transferred to NH_4Cl-deficient media, and harvested at 3, 6, and 9 h after depletion. Relative GSH content indicates a GSH value of the treated culture compared with the corresponding control value.

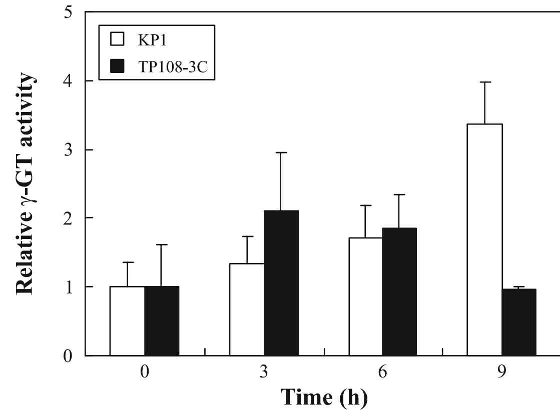

Fig. 4. Variations in γ-GT activity of Pap1-positive *S. pombe* KP1 and Pap1-negative *S. pombe* TP108-3C cells during nitrogen starvation. Each column represents Mean±SE The yeast cultures were transferred to NH_4Cl-deficient media, and harvested at 3, 6, and 9 h after depletion. Relative γ-GT activity indicates a γ-GT value of the treated culture compared with the corresponding control value.

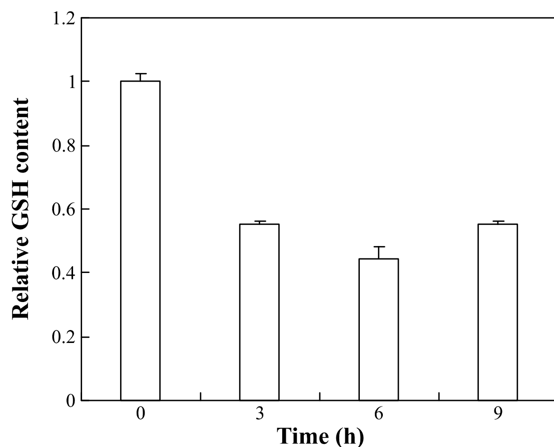

Fig. 3. Effect of glucose depletion on total GSH contents in Pap1-positive *S. pombe* KP1 cells during nitrogen starvation. Each column represents Mean±SE The yeast culture was transferred to glucose-deficient media, and harvested at 3, 6, and 9 h after depletion. Relative GSH content indicates a GSH value of the treated culture compared with the corresponding control value.

transferred to a nitrogen-deficient medium, they stop vegetative growth and initiation of sexual development. Accordingly, nitrogen depletion gives rise to variations in expression of various defense-related genes. GSH is present in high concentrations of up to 10 mM in yeasts. When the *S. cerevisiae* cells were subjected to nitrogen starvation, the total GSH pool significantly increased during the first 2 h and then declined, while more than 90% of the cellular GSH shifted toward the central vacuole of the yeast (Mehdi and Penninckx, 1997). Although short-term fasting followed by a refeeding period which decreased the concentrations of various amino acids in human muscle and plasma, no changes were observed in the GSH levels and their redox status (Hammarqvist *et al.*, 2005). The GSH content in Pap1-positive KP1 cells markedly increased up to 6 h after the depletion of nitrogen and slightly declined after 9 h (Fig. 2). However, an increase in the GSH content was detected to be much less in Pap1-negative TP108-3C cells during nitrogen starvation (Fig. 2). The yeast growth slightly decreased during nitrogen starvation in both Pap1-positive and Pap1-negative cells (data not shown). Deprivation of glucose as a sole carbon source caused a decrease in the GSH content of Pap1-positive KP1 cells (Fig. 2). However, deprivation of glucose completely arrested the growth of the yeast cells (data not shown). Collectively, nitrogen starvation causes up-regulation of the GSH content in *S. pombe* in a Pap1-dependent fashion.

Nitrogen starvation causes up-regulation of γ-GT

γ-GT, which is a membrane-bound enzyme ubiquitously distributed in living organisms, catalyzes the degradation of GSH by cleavage of the γ-glutamyl bond, allowing the supply of extracellular cysteine for intracellular synthesis of GSH. In *S. cerevisiae*, the *CIS2* gene encoding γ-GT was mainly induced by nitrogen starvation and poor nitrogen sources such as urea (Springael and Penninckx, 2003).

γ-GT induced during nitrogen starvation was translocated from the Golgi toward the vacuolar membrane, which could make the starved yeast cells use the constituent amino acids from GSH accumulated in the vacuole to satisfy their requirements for nitrogen (Penninckx, 2002). Since GSH content in the *S. pombe* cells was increased by nitrogen depletion, effect of nitrogen depletion on γ-GT activity was also subsequently examined in *S. pombe* cells. The γ-GT activity in Pap1-positive KP1 cells was enhanced by nitrogen depletion increasing approximately 3-fold after 9 h (Fig. 4). Compared to the maximum value of GSH content at 6 h, γ-GT activity increased at a later stage after the depletion. This might suggest that γ-GT activity is physiologically required to metabolize the increased GSH under nitrogen starvation. However, the precise reason for the time difference in the up-regulation of GSH and γ-GT remains currently

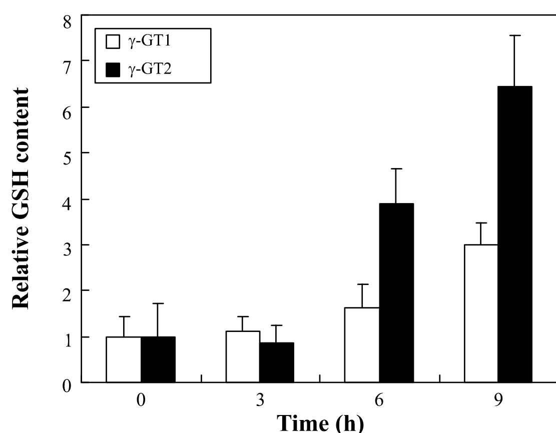

Fig. 5. Variations in total GSH contents of Pap1-positive *S. pombe* KP1 cells overexpressing γ-GT1 or γ-GT2 during nitrogen starvation. Each column represents Mean±SE The yeast cultures were transferred to NH_4Cl-deficient media, and harvested at 3, 6, and 9 h after depletion. Relative GSH content indicates a GSH value of the treated culture compared with the corresponding control value.

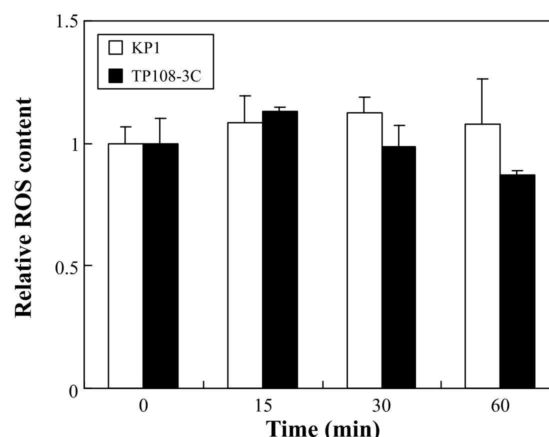

Fig. 7. Variations in ROS levels of Pap1-positive *S. pombe* KP1 and Pap1-negative *S. pombe* TP108-3C cells during nitrogen starvation. The yeast cultures were transferred to NH_4Cl-deficient media, and harvested at 3, 6, and 9 h after depletion. The ROS levels were determined as described in 'Materials and Methods'. Relative ROS content indicates an ROS value of the treated culture compared with the corresponding control value.

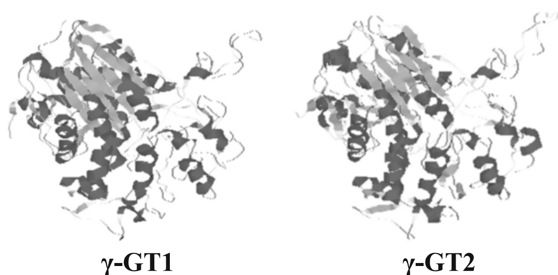

Fig. 6. Predictive three-dimensional structures of γ-GT1 and γ-GT2 from *S. pombe*. The three-dimensional structures were estimated by RasMol program.

unknown. The Pap1-negative TP108-3C cells also showed a similarly increasing pattern during nitrogen starvation, but significantly declined at 9 h (Fig. 4).

In previous studies, the two *γ-GT* genes, *γ-GT1* and *γ-GT2*, were cloned and characterized from *S. pombe* (Kang *et al*., 2005; Kim *et al*., 2005). Nitrogen starvation was able to induce both *γ-GT1* and *γ-GT2* genes in Pap1-independent and Pap1-dependent manner, respectively, This might suggest that γ-GT2 is more appropriate than γ-GT1 in adapting to nitrogen starvation, although the precise role of γ-GT2 remains uncertain. The Pap1-positive cells overexpressing γ-GT2 showed significantly higher GSH content than the yeast cells overexpressing γ-GT1 (Fig. 5). This difference might link with the fact that the *γ-GT2* gene is only under Pap1 control during nitrogen starvation. The predictive three-dimensional structures of the *S. pombe* γ-GT1 and γ-GT2 appeared to resemble each other (Fig. 6).

Reactive oxygen species level

ROS at physiological concentrations required for normal cellular function is involved in intracellular signaling and redox regulation (Nordberg and Arnér, 2001). ROS at excessive level leads to oxidative stress, which threatens the integrity of various biomolecules and subsequently is involved in the process of apoptosis and aging. Therefore, ROS level is very crucial in both cellular growth and death as well as stress response. The ROS levels were not significantly changed by nitrogen depletion in both KP1 and TP108-3C cells (Fig. 7). Recently, autophage gene analogs have been shown to be involved in responses against nitrogen depletion. The fission yeast undergoes autophage as a bulk protein degradation process induced by the deprivation of exogenous nitrogen, which supplies nitrogen (Kohda *et al.*, 2007). Under nitrogen starvation, protein degradation is dependent upon autophage gene orthologs, such as *atg1*, *atg8*, and *atg13* (Kohda *et al.*, 2007). Atg1 and Atg13 are known to be dephosphorylated under depletion of nitrogen. Some of the autophage gene analogs might also participate in the up-regulation of GSH under nitrogen starvation. In conclusion, this finding may suggest that ROS is not involved in up-regulation of GSH content and γ-GT by nitrogen starvation in *S. pombe*.

Acknowledgements

This work was supported by a grant (No. R01-2003-000-10029-0) from the Basic Research Program of the Korea Science and Engineering Foundation.

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