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Running title: Regulation of GPX1 expression

Regulatory mechanism for expression of GPX1 in response to glucose starvation and Ca^{2+} in Saccharomyces cerevisiae: Involvement of Snf1 and Ras/cAMP pathway in Ca^{2+} signaling

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Saccharomyces cerevisiae has three homologues of the glutathione peroxidase gene, GPX1, GPX2, and GPX3. We have previously reported that the expression of GPX3 was constitutive, but that of GPX2 was induced by oxidative stress and CaCl2, and uncovered the regulatory mechanisms involved. Here we show that the expression of GPX1 is induced by glucose starvation and treatment with CaCl2. The induction of GPX1 expression in response to glucose starvation and Ca2+ was dependent on the transcription factors Msn2 and Msn4 and cis-acting elements (stress response element, STRE) in the GPX1 promoter. The Ras/cAMP pathway is also involved in the expression of GPX1. We found that Snf1, a Ser/Thr protein kinase, is involved in the glucose starvation- and Ca2+-induced expression of GPX1. The activation of Snf1 is accompanied by phosphorylation of Thr210. We found that the Ca2+-treatment as well as glucose starvation causes the phosphorylation of Thr210 of Snf1 in a Tos3, Sak1, and Elm1 protein kinase-dependent manner. Since the timing of the initiation of the Ca2+-induced expression of GPX1 was retarded in an snf1Δ mutant, the activation of Snf1 seems pivotal to the early-stage-response of GPX1 to Ca2+. 
Introduction

Cells alter their pattern of gene expression in response to environmental stress, such as oxidative stress, heat shock stress and osmotic stress. The change in the availability of nutrients, such as amino acids and glucose, is also crucial to the remodeling of gene expression. The budding yeast *Saccharomyces cerevisiae* has provided an excellent model with which to analyze the regulatory mechanism of gene expression as well as cell signaling systems. Cellular responses to glucose have also been studied extensively using yeasts as models. In the presence of glucose, two Ras proteins, Ras1 and Ras2, activate adenylate cyclase. The activated adenylate cyclase catalyzes the synthesis of cAMP, which in turn activates the protein kinase A (PKA) pathway (Gancedo 1998; Carlson 1999; Rolland *et al.* 2002). PKA is involved in many important cellular events, such as gene expression, cell cycle control, and stress response (Santangelo 2006). Regarding the response to stress, PKA regulates the nucleocytoplasmic localization of the transcription factors Msn2 and Msn4 (Görner *et al.* 1998). Msn2 and Msn4, C$_2$H$_2$ zinc-finger proteins, are known to be important in responses to various types of environmental stress (Estruch 2000). Msn2 and Msn4 bind to a characteristic DNA sequence referred to as the stress response element (STRE, 5’-AGGGG-3’ or 5’-CCCCT-3’) in the promoter region of various stress-responsive genes, and induce their expression. PKA phosphorylates Msn2, thereby sequestering it to the cytoplasm (Görner *et al.* 2002). Therefore, the low level of PKA activity is associated with an increasing proportion of nuclear Msn2, leading to transcriptional activation.

Glucose repression is a well-known phenomenon in not only yeasts but also many organisms. Snf1, a Ser/Thr protein kinase and a tentative counterpart of a mammalian AMP-activated protein kinase (AMPK), is a key regulator for the derepression of the glucose-repressed genes in yeast (Gancedo 1998; Carlson 1999).
Thr$^{172}$ of mammalian AMPK is phosphorylated by a Ca$^{2+}$/calmodulin-dependent protein kinase kinase (CaMKK) during the activation process (Hawley et al. 2005; Hurley et al. 2005; Woods et al. 2005). In the activation of Snf1 in cells starved of glucose, Thr$^{210}$ of Snf1 is phosphorylated by three redundant upstream protein kinases, Tos3, Elm1, and Sak1, homologues of mammalian LKB1 and CaMKKα (Hong et al. 2005). Snf1 thus activated phosphorylates its target such as Mig1 (Treitel et al. 1998).

The availability of nitrogen also affects many cellular events. The target of rapamycin (TOR) pathway plays crucial roles in nitrogen signaling in yeasts and mammals (De Virgilio & Loewith 2006). The pathway regulates transcription, translation, ribosome biogenesis, nutrient transport, autophagy, and the organization of the actin cytoskeleton (Schmelzle & Hall 2000). Regarding the correlation between stress responses and the TOR pathway, it has been proven that TOR kinase negatively regulates the nuclear localization of Msn2 and Msn4 (Beck & Hall 1999).

When starved of nutrient, yeast cells undergo a remodeling of metabolism and gene expression to survive extended periods: accumulating storage carbohydrates such as glycogen and glycerol, increasing the expression of stress-responsive genes, and thickening the cell wall (Herman 2002; Longo et al. 1996). Oxidative stress is one of the most notable features in the stationary phase, in which the availability of nutrients would be limited. Organisms are armed with several antioxidants to scavenge reactive oxygen species, such as superoxide dismutase, catalase, and peroxidases. The expression of yeast CTT1 (cytosolic catalase), TSA2 (cytosolic thiol peroxidase), and PRX1 (mitochondrial thiol peroxidase) is induced by glucose starvation in an Msn2 and Msn4-dependent manner (Hong et al. 2002; Martinez-Pastor et al. 1996; Monteiro & Netto 2004). We have previously cloned the GPX1, GPX2, and GPX3 genes in S. cerevisiae encoding a homologue of mammalian glutathione peroxidase (GPx), a major antioxidant in higher eukaryotes (Inoue et al. 1999).
In contrast to Gpx2 and Gpx3, information about Gpx1 has been largely lacking. To gain a clue as to the physiological function of Gpx1, here we searched for the conditions that induce the expression of GPX1, and investigated the regulatory mechanism involved. We found that the expression of GPX1 was induced by glucose starvation, nitrogen starvation, and treatment with CaCl₂ in an Msn2 and Msn4-dependent manner. The Ras/cAMP pathway, TOR pathway and Snf1 kinase were involved in the upstream modules for the glucose starvation-induced expression of GPX1. Notably, we found that Snf1 is involved in the Ca²⁺ signaling pathway. Hence, in the present study, we aimed to clarify the regulatory mechanism of the expression of GPX1, and also tried to show the role of Snf1 in Ca²⁺ signaling through the elucidation of the expression of GPX1.

**Results**

**Expression of GPX1 is induced by glucose starvation**

We have previously reported that expression of GPX1 is induced when logarithmically growing cells in YPD medium are transferred to glucose-starved medium (Inoue et al. 1999). To quantify the expression of GPX1, a GPX1-lacZ reporter gene was constructed. As shown in Fig. 1A, mRNA levels of GPX1 increased upon glucose starvation as we reported previously (Inoue et al. 1999). β-Galactosidase activity derived from GPX1-lacZ was also increased in glucose-starved cells. We constructed a HA-tagged GPX1 gene (GPX1-3HA) also to monitor the Gpx1 protein level. As shown in Fig. 1A, Gpx1 protein levels also increased with glucose starvation. Collectively, the monitoring systems (GPX1-lacZ and GPX1-3HA) constructed here are valid to evaluate the expression of GPX1.
Expression of GPX1 is induced by Ca$^{2+}$

By using GPX1-lacZ, we screened the conditions that induce the expression of GPX1, and found that the expression was induced following treatment with CaCl$_2$. The maximum induction of GPX1-lacZ expression in terms of the CaCl$_2$ concentration was attained at 400 mM (data not shown). As shown in Fig. 1A, mRNA levels of GPX1 as well as protein levels of Gpx1 also increased following treatment with CaCl$_2$.

High concentrations of CaCl$_2$ cause an osmotic stress response in yeast. However, the expression of GPX1 was not substantially induced by osmotic stress caused by 1 M sorbitol (data not shown). Hog1, a mitogen-activated protein (MAP) kinase responsible for the osmotic stress response in S. cerevisiae (Brewster et al. 1993; Errede & Levin 1993), was phosphorylated and accumulated in the nucleus following treatment with 300 mM CaCl$_2$ (Takatsume, Y., Ohdate, T., Maeta, K., Nomura, W., Izawa, S., and Inoue, Y., submitted). To determine whether Hog1 is involved in the Ca$^{2+}$-induced expression of GPX1, HOG1 was disrupted. As a result, expression of GPX1 was induced in hog1Δ cells following treatment with CaCl$_2$ when evaluated using GPX1-lacZ (Fig. 1B), indicating that the Hog1-MAP kinase pathway is not involved in the Ca$^{2+}$-induced expression of GPX1. Hence, the induction of the expression of GPX1 in response to Ca$^{2+}$ seems not a response to osmotic stress.

The calcineurin/Crz1 pathway is a major device of Ca$^{2+}$ signaling in yeast (Cohen 1989; Cyert 2003; Sugiura et al. 2001; Yoshimoto et al. 2002). A gene whose expression is regulated by the calcineurin/Crz1-mediated pathway has a distinct DNA sequence called CDRE (calcineurin-dependent response element). Yoshimoto et al. (2002) have reported a genome-wide analysis of gene expression that is regulated in a calcineurin/Crz1-dependent manner in S. cerevisiae. The computational analysis revealed that the calcineurin/Crz1-dependent genes contain a common sequence
(5’-GAGGCTG-3’) in their promoter region (Yoshimoto et al. 2002). We have previously reported that the expression of \( \text{GPX2} \), another gene of GPx homologue in \( S. \text{cerevisiae} \), was induced following treatment with \( \text{CaCl}_2 \) in a calcineurin/Crz1-dependent manner (Tsuzi et al. 2004b). Thus, we looked for this motif in the \( \text{GPX1} \) promoter, although no such a sequence was found. Indeed, the induction of \( \text{GPX1} \) expression following treatment with \( \text{Ca}^{2+} \) did occur even in a mutant lacking Crz1 (Fig. 1B).

**Msn2 and Msn4 act as key transcription factors of \( \text{GPX1} \)**

The \( \text{GPX1} \) promoter has two putative STREs (consensus sequence: 5’-CCCCT-3’ or 5’-AGGGG-3’) located between -303 and -298 (STRE1; 5’-CCCCT-3’), and between -248 and -243 (STRE2; 5’-CCCCT-3’) upstream of the translation initiation codon of \( \text{GPX1} \) (Fig. 2A). Msn2 and Msn4 are known to be responsible for general stress responses, and bind to STRE to induce the expression of a wide variety of stress-responsive genes. Although the \( \text{GPX2} \) promoter has the consensus sequence of STRE, neither Msn2 nor Msn4 regulates the expression of \( \text{GPX2} \) under oxidative stress and \( \text{CaCl}_2 \)-treatment (Tsuzi et al. 2004a, 2004b). To determine whether Msn2 and Msn4 are involved in the expression of \( \text{GPX1} \) induced by glucose starvation and \( \text{Ca}^{2+} \), we disrupted \( \text{MSN2} \) and \( \text{MSN4} \). The induction of \( \text{GPX1} \) expression under glucose starvation and \( \text{Ca}^{2+} \)-treatment was vastly repressed in \( \text{msn2} \Delta \text{msn4} \Delta \) cells (Fig. 2B). Therefore, Msn2 and Msn4 are key transcription factors in the expression of \( \text{GPX1} \).

**Roles of two putative STREs in \( \text{GPX1} \) promoter as cis-element**

To determine whether the two STREs in the \( \text{GPX1} \) promoter function as cis-elements in response to glucose starvation and \( \text{Ca}^{2+} \) signaling, the consensus sequence of STRE1 and/or STRE2 was changed to 5’-CATCT-3’ (Fig. 2A), the mutation which was proven to disable STRE’s function (Zahringer et al. 2000). Each STRE-mutated \( \text{GPX1} \) promoter
was fused with the lacZ gene, and the resulting reporter genes (STRE1mut-, STRE2mut-, and STRE1/2mut-lacZ) were integrated into the genome of S. cerevisiae. All STRE mutations resulted in a marked decrease in the GPX1 promoter activity in response to glucose starvation and CaCl₂ (Fig. 2C).

To verify further whether the STREs found in the GPX1 promoter function as the cis-acting elements in terms of glucose-starvation as well as Ca²⁺-induced responses, the corresponding region (-345 to -182) was introduced upstream of the CYC1 TATA-lacZ reporter gene in pTBA30 (Galius-Duerner et al. 1997). As shown in Fig. 2D, the β-galactosidase activity derived from pGPX1-CYC1-lacZ was increased by glucose starvation and Ca²⁺ as observed for GPX1-lacZ, which has the original promoter of GPX1. When pGPX1-CYC1-lacZ was introduced into the msn2∆msn4∆ mutant, no increase in β-galactosidase activity was observed in response to glucose starvation or CaCl₂ treatment (Fig. 2D). Taken together, Msn2 and Msn4 are responsible for the glucose starvation and Ca²⁺-induced signaling response of GPX1, and STREs in the GPX1 promoter function as the cis-acting elements for such events.

Role of Ras/cAMP pathway in expression of GPX1

The Ras/cAMP pathway negatively regulates the nucleocytoplasmic localization of Msn2 and Msn4 via PKA (Görner et al. 1998). We determined the expression of GPX1 in ras2Δ cells in response to glucose starvation and CaCl₂. As we expected, the deletion of RAS2 enhanced the basal level of Gpx1 protein under non-stressed conditions. The glucose starvation and CaCl₂ treatment increased Gpx1 protein levels further even in ras2Δ cells (Figs. 3A and 3B). So, first, we explored the machinery involved in the Ca²⁺-induced expression of GPX1 in ras2Δ cells. Since we have demonstrated that Msn2 and Msn4 play crucial roles in the Ca²⁺-induced expression of GPX1, and the PKA pathway negatively regulates Msn2 and Msn4 (Görner et al. 1998), we determined whether or not
the increase in the basal level of GPX1 expression in ras2Δ cells is suppressed by the disruption of MSN2 and MSN4. As shown in Fig. 3B, basal level the GPX1-lacZ expression in msn2Δmsn4Δras2Δ cells was reverted to that in wild type cells. Next, to verify the involvement of the Ras/cAMP pathway in the expression of GPX1 in response to Ca2+, the phosphorylation state of Msn2 was determined. It has been reported that Ser582 and Ser620 of Msn2 are phosphorylated by PKA, thereby sequestering this transcription factor in the cytoplasm when enough glucose is supplied in the medium (Görner et al. 2002). We confirmed that Msn2 is concentrated in cells starved of glucose (Fig. 3C). We have previously reported that Msn2 is concentrated in the nucleus in cells treated with CaCl2, which was also confirmed in this study (Fig. 3C). In ras2Δ cells, Msn2 was partially distributed in the nucleus even though enough glucose is present, although upon glucose starvation or CaCl2-treatment, nuclear Msn2 was clearly observed (Fig. 3C). It has been reported that these two phospho-serines are dephosphorylated upon glucose starvation, which was confirmed in this study (Fig. 3D). As shown in Fig. 3D, both Ser582 and Ser620 of Msn2 in wild-type cells were slightly dephosphorylated following treatment with CaCl2 also. These results suggest that Ca2+ gives rise to a negative effect on the PKA pathway.

Since Gpx1 protein levels increased further in ras2Δ cells starved of glucose, in which cells Msn2 and Msn4 may be activated (Fig. 3A) even in the presence of glucose, in the next paragraph we explored another machinery involved in the glucose starvation- and Ca2+-induced expression of GPX1.

**Effect of Snf1 kinase on expression of Gpx1**

Snf1, a Ser/Thr protein kinase, is one of the key regulators for the derepression of glucose-repressed genes in yeast. To determine whether Snf1 is involved in the expression of GPX1, we examined the Gpx1 level in an snf1Δ mutant. In wild type cells,
Gpx1 level increased after 30 min upon glucose starvation, and the level was kept almost constant until 180 min (Fig. 4A). In snf1Δ cells, Gpx1 level was also increased after 30 min of glucose starvation, and the level was almost constant within the period of experiment; however, overall level of Gpx1 in snf1Δ cells was lower compared with that of wild type cells (Fig. 4A). By contrast, in wild type cells Gpx1 level began to increase after 30 min of CaCl2 treatment, peaked at 60 min, and then declined. Whereas in snf1Δ cells, intriguingly, the beginning of the Ca2+-induced expression of GPX1 was retarded for 30 min compared with that in wild type cells (Fig. 4A). The Gpx1 level reached a maximum after 90 min of CaCl2 treatment, and it decreased slowly compared with that of wild type. These results suggest the following two possibilities: (i) Snf1 is involved in the early-stage-response (~30 min) of GPX1 to Ca2+, and (ii) Snf1 is involved in turnover of Gpx1 protein in the presence of Ca2+.

**Snf1 is involved in Ca2+ signaling pathway to GPX1**

Next, we determined the effect of Ca2+ on phosphorylation of Snf1. In the activation of Snf1 in cells starved of glucose, Thr210 is phosphorylated (McCartney & Schmidt 2001, also see Fig. 4B). So, we determined whether Thr210 of Snf1 is phosphorylated upon treatment with CaCl2. As shown in Fig. 4B, Thr210 was phosphorylated following treatment with CaCl2.

Three upstream protein kinases, Tos3, Elm1 and Sak1, have been identified to phosphorylate Snf1 (Hong et al. 2003; Nath et al. 2003; Sutherland et al. 2003). In a tos3Δelm1Δsak1Δ mutant, Snf1 was not phosphorylated at all by glucose starvation (Hong et al. 2003), which was confirmed in this study (Fig. 4B). We determined whether Snf1 is activated by treatment with CaCl2 in the tos3Δelm1Δsak1Δ mutant. As shown in Fig. 4B, Snf1 phosphorylation did not occur in this mutant. Nonetheless, the phosphorylation level of Snf1 in cells treated with CaCl2 was slightly lower compared
with that in cells starved of glucose. Calcineruin is a protein phosphatase that is activated by \( \text{Ca}^{2+}/\text{calmodulin} \) (Cohen 1989; Cyert 2003), following treatment with \( \text{CaCl}_2 \). To address the possibility that calcineruin dephosphorylates Snf1 in the presence of \( \text{Ca}^{2+} \), we determined the phosphorylation level of Snf1 in a mutant defective in calcineurin (\( \text{cnb1}\Delta \)). As shown in Fig. 4C, no distinct differences in the phosphorylation states of Snf1 in cells starved of glucose or treated with \( \text{CaCl}_2 \) were observed between wild type and \( \text{cnb1}\Delta \) mutant. So, calcineurin is not likely to be involved in the control of phosphorylation state of Snf1 in the presence of \( \text{Ca}^{2+} \).

Next, we determined the nucleocytoplasmic localization of Snf1. Snf1 distributes in the cytoplasm when glucose is supplied sufficiently, but upon glucose starvation, it is concentrated in the nucleus (Vincent \textit{et al.} 2001). We confirmed the nuclear accumulation of Snf1 in cells starved of glucose (Fig. 4D). Indirect immunofluorescence microscopy revealed that Snf1 is concentrated in the nuclei in wild-type cells treated with \( \text{CaCl}_2 \); however, in the \( \text{tos3}\Delta\text{elm1}\Delta\text{sak1}\Delta \) mutant, Snf1 resided in the cytoplasm (Fig. 4D). Together with the phosphorylation of Snf1, three upstream protein kinases (Tos3, Elm1 and Sak1) are activated following treatment with \( \text{CaCl}_2 \), thereby phosphorylating Snf1, which allows Snf1 to accumulate in the nucleus.

**Chronic response of \( \text{GPX1} \) to glucose-limited conditions**

Under the experimental conditions thus far employed, yeast cells at a log phase of growth were suddenly transferred to a glucose-starved (0.1% glucose) medium. Next, we determined whether the expression of \( \text{GPX1} \) was also induced when the glucose level in the medium was decreased gradually in accordance with the growth of yeast cells. So, the amount of Gpx1 protein in the cells cultured in YPD medium was monitored periodically. The glucose concentration in the medium was also measured. The level of Gpx1 reached a plateau after 37 h from the beginning of the culture, and concomitantly, the glucose in
the medium was depleted after 33-37 h (Fig. 5A). The diauxic shift is defined as the period in which yeast cells consume glucose in the medium completely, and change from fermentation to respiration as a means to obtain energy. The Gpx1 level was high during the diauxic shift (37-49 h), and it was kept constant in the post-diauxic shift (61-73 h).

**Contribution of Gis1 to the post-diauxic response of GPX1**
The chronic response of Gpx1 was monitored using the msn2Δmsn4Δ mutant also (Fig. 5B). Basically, the level of Gpx1 in msn2Δmsn4Δ cells was low compared with that in wild-type cells, although immunoreactive bands corresponding to Gpx1-3HA were observed even in msn2Δmsn4Δ cells at the diauxic shift. Therefore, other transcription factor(s) may be involved in the expression of GPX1 in the stationary phase. Pedruzzi et al. (2000) have reported that the transcription factor Gis1, responsible for genes induced post-diauxic shift, can partially mediate the induction of some genes possessing STRE. Hence, we constructed an msn2Δmsn4Δgis1Δ mutant, and analyzed the expression of GPX1-3HA by Western blotting. The level of Gpx1 protein at the diauxic shift in a gis1Δ mutant was comparable with that in the wild type (data not shown), although no Gpx1 protein was observed in the msn2Δmsn4Δgis1Δ mutant (Fig. 5B). Since the induction of Gpx1 in response to glucose starvation and Ca^{2+}-treatment occurred in gis1Δ cells at the log phase of growth (data not shown), Gis1 seems to contribute little in the response of GPX1 to stress, but does contribute partially to the post-diauxic response of this gene.

**Response of GPX1 to nitrogen starvation**
We have demonstrated that the expression of GPX1 is induced by glucose-starvation as well as entry into the stationary phase, where not only glucose but also nitrogen sources may be limited. So, we next determined whether the expression of GPX1 is induced when nitrogen is limited. The Gpx1 protein level was monitored at 6, 12 and 24-h after transfer
to a nitrogen-starved medium, because protein synthesis under such conditions may proceed slowly due to the limited amount of nitrogen sources such as amino acids. As a result, the amount of Gpx1 in cells shifted to the nitrogen-starved medium was increased (Fig. 6A). The level of Gpx1 was also increased when cells were cultured in a nitrogen-sufficient medium, which is due to the decrease in glucose in the medium. Glucose in the nitrogen-starved medium was consumed slowly compared with the medium supplemented with a sufficient amount of nitrogen; nonetheless, the Gpx1 level increased to a much greater extent in cells cultured in the nitrogen-starved medium than in cells in the nitrogen-sufficient medium. Therefore, we concluded that the expression of GPX1 is induced by nitrogen starvation also.

The TOR pathway regulates the expression of GPX1

To gain an insight into the regulatory mechanism behind the nitrogen starvation-induced expression of GPX1, we examined whether the TOR pathway, which is mainly controlled by nitrogen, is involved. We have demonstrated that Msn2 and Msn4 play crucial roles in the induction of GPX1 expression. It has been reported that these transcription factors are negatively regulated by the TOR pathway by being sequestered in the cytoplasm (Beck & Hall 1999). *S. cerevisiae* has two TOR kinases, Tor1 and Tor2 (Heitman *et al.* 1991). The two TOR kinases in TORC1 (TOR complex I) have a redundant function in, for example, regulating transcription, translation, ribosome biogenesis and autophagy; whereas, TORC2 (TOR complex II), where only Tor2 is involved, has a unique function in regulating the organization of the actin cytoskeleton (Schmelzle & Hall 2000). To determine whether the TOR pathway is involved in expression of the GPX1 gene in response to starvation, cells were treated with rapamycin, a potent inhibitor of TORC1 (Heitman *et al.* 1991). As shown in Fig. 6B, the mRNA levels of GPX1 were greatly increased following treatment with rapamycin. Therefore, the TOR pathway negatively
regulates the expression of \textit{GPX1}.

**Role of Gpx1 under glucose starved conditions**

To explore the physiological relevance to induction of \textit{GPX1}, we determined the capability of wild type and \textit{gpx1Δ} cells, which had been adapted to glucose-limited conditions, to grow in the presence of H$_2$O$_2$ in glucose-starved medium. As shown in Fig. 7A, the growth of a \textit{gpx1Δ} mutant in the glucose-starved medium containing H$_2$O$_2$ was impaired compared with that of wild type. Similarly, cell growth of the \textit{msn2Δmsn4Δ} mutant was also impaired under the same conditions. We examined the growth of yeast cells in the presence of both Ca$^{2+}$ and H$_2$O$_2$, although no additive effect of Ca$^{2+}$ on the H$_2$O$_2$-induced growth defect of \textit{gpx1Δ} and \textit{msn2Δmsn4Δ} mutants was observed (data not shown).

On the other hand, we have shown that the Gpx1 level increased in the post-diauxic shift (Fig. 5A). Glycerol medium is sometimes used to mimic the conditions of post-diauxic shift (Roosen \textit{et al.} 2005), so we monitored the growth of yeast cells in glycerol medium. As shown in Fig. 7B, no substantial difference was observed in the generation time of wild type, \textit{gpx1Δ}, and \textit{gpx1Δgpx2Δgpx3Δ} cells in the log-phase of growth in the glycerol medium, although maximum growth of the \textit{gpx1Δ} mutant and \textit{gpx1Δgpx2Δgpx3Δ} mutant was lower by approximately 20\% and 30\%, respectively, compared with that of wild type.

**Discussion**

**Regulatory mechanism for expression of \textit{GPX1} in response to glucose starvation and Ca$^{2+}$**
In the present study, we found that the expression of \textit{GPX1} in \textit{S. cerevisiae} is induced by not only glucose starvation, which we have reported previously (Inoue \textit{et al.} 1999), but also by CaCl$_2$ treatment, and investigated the regulatory mechanisms involved. The C$_2$H$_2$ zinc-finger transcription factors Msn2 and Msn4, and two \textit{cis}-acting elements (STREs) in the \textit{GPX1} promoter played crucial roles. Snf1 kinase was also involved in response to both glucose starvation and Ca$^{2+}$ of \textit{GPX1}.

The Mig1–Ssn6–Tup1 global complex is the major repressor of glucose-repressed genes, which binds to the upstream repression site (URS) of the respective genes, when glucose is present (Treitel \textit{et al.} 1998). When glucose is limited, Snf1 is activated and phosphorylates Mig1, thereby dissociating the repressor complex form URS, and consequently, glucose repression is cancelled to induce the expression of glucose-repressive genes (Gancedo 1998; Carlson 1999). First, we suspected that Mig1 is involved in the glucose-starvation-induced expression of \textit{GPX1}, because Mig1 binds to the G cluster preceded by the AT-rich sequence (Lundin \textit{et al.} 1994), and the \textit{GPX1} promoter contains such a sequence (5’-TTAAAGCCGGGG-3’) 182-bp upstream of the translational initiation codon. We then disrupted \textit{MIG1} and its parologue \textit{MIG2}, however, the basal expression level of \textit{GPX1} did not increase in such mutants (data not shown). This was also the case in \textit{ssn6}Δ and \textit{tup1}Δ cells (data not shown). In addition, the glucose starvation-induced expression of \textit{GPX1} occurred in these mutants (data not shown); and therefore, the Mig1–Ssn6–Tup1 global repressor seems not involved in the regulatory mechanism of the expression of \textit{GPX1} in response to glucose starvation. Nonetheless, since the glucose starvation-induced expression of \textit{GPX1} did not occur sufficiently in \textit{snf1}Δ cells (Fig. 4A), it is conceivable that Snf1 plays some important role in induction of the expression of \textit{GPX1} under glucose-limited conditions, rather than in derepression of glucose repression of \textit{GPX1}.

Both Ser$^{582}$ and Ser$^{620}$ of Msn2 are dephosphorylated in response to glucose
starvation (De Wever *et al.* 2005; Görner *et al.* 1998), which was confirmed in this study (Fig. 3D). These two serine residues are believed to be phosphorylated by PKA, and their phosphorylation state is correlated with the nucleocytoplasmic localization of Msn2 (Görner *et al.* 1998). Here we showed that these serine residues are dephosphorylated in response to Ca\(^{2+}\). Therefore, Ca\(^{2+}\) may partially regulate PKA activity negatively.

Msn2 is concentrated in the nucleus by a wide variety of environmental stresses such as heat shock, osmotic stress, and oxidative stress, and such stress signals converge on the nuclear export signal (NES) at the N terminus of Msn2, which causes the phosphorylation of several serine residues in the NES to block the nuclear export of Msn2 (Görner *et al.* 2002). Hyperphosphorylation of Msn2 is observed when cells are challenged by heat shock (Garreau *et al.* 2000). On the other hand, Ser\(^{582}\) and Ser\(^{620}\) lie within the nuclear localization signal (NLS) of Msn2, and the phosphorylation of these serine residues by PKA inhibits the nuclear import of Msn2 (Görner *et al.* 2002). We have previously reported that Msn2 is concentrated in the nucleus following treatment with CaCl\(_2\) (Tsuzi *et al.* 2004b), but Msn2 was not hyperphosphorylated upon treatment with CaCl\(_2\), instead Ser\(^{582}\) and Ser\(^{620}\) were dephosphorylated (Fig. 3D). In ras2\(\Delta\) cells, the steady state activity of PKA is expected to be lowered due to the insufficient supply of cAMP, which would lead to dephosphorylation of Ser\(^{598}\) and Ser\(^{620}\) of Msn2, and consequently Msn2 is activated. Indeed, Msn2 was partially distributed in the nucleus in ras2\(\Delta\) cells even though enough glucose is supplied (Fig. 3C). Therefore, the observation that the basal level of Gpx1 protein was increased in the ras2\(\Delta\) mutant is rational (Figs. 3A and 3B). Nonetheless, we found that Msn2 was concentrated in the nucleus in ras2\(\Delta\) cells following treatment with CaCl\(_2\), and consequently, the levels of Gpx1 were further increased (Figs. 3A-3C). Therefore, Ca\(^{2+}\) signal is sent to Msn2 besides deactivation of the PKA pathway, thereby activating the transcription of GPX1 (Fig. 8).

A striking feature of the regulatory mechanism of GPX1 is that Snf1 was
involved not only in the glucose starvation-induced but also in the Ca\textsuperscript{2+}-induced expression of \textit{GPX1}. Intriguingly, here we first demonstrated that Thr\textsuperscript{210} in Snf1 is phosphorylated following treatment with CaCl\textsubscript{2} in a Tos3, Elm1, and Sak1 protein kinase-dependent manner. These three protein kinases are homologues of mammalian CaMKK\textalpha{}, a Ca\textsuperscript{2+}/calmodulin-dependent kinase kinase, but it has never been examined whether Ca\textsuperscript{2+} activates them in yeast. Since Tos3, Elm1, and Sak1 are homologues of CaMKK\textalpha{}, it is conceivable that Ca\textsuperscript{2+} activates them in yeast. Recently, Hong and Carlson (2007) have reported that Snf1 is phosphorylated in cells challenged by Na\textsuperscript{+} or alkaline stress. Denis and Cyert (2002) have demonstrated that Ca\textsuperscript{2+} in vacuole is released into the cytoplasm upon exposure of yeast cells to Na\textsuperscript{+}. Meanwhile, alkaline stress induces the uptake of extracellular Ca\textsuperscript{2+} to bind calmodulin, thereby activating calcineurin (Viladevall et al. 2004). Additionally, mammalian CaMKK\textalpha{} is able to phosphorylate Thr\textsuperscript{210} of Snf1 in a tos3\textDelta{}elm1\textDelta{}sak1\textDelta{} mutant following treatment with NaCl or alkaline stress (Hong & Carlson 2007). Together these earlier reports, our data obtained in the present study suggest that an increase in the level of Ca\textsuperscript{2+} in cells is a trigger of the activation of Snf1 via environmental stress.

Even though Snf1 is activated following treatment with CaCl\textsubscript{2}, the Gpx1 level increased in \textit{snf1\textDelta{}} cells in the presence of Ca\textsuperscript{2+} (Fig. 4A). However, the timing of the initiation of the Ca\textsuperscript{2+}-induced expression was delayed for 30 min in the \textit{snf1\textDelta{}} mutant (Fig. 4A). Therefore, Ca\textsuperscript{2+} signal seems to be sent to \textit{GPX1} through three different routes; i.e. (i) Ca\textsuperscript{2+} gives rise to a negative effect on to the Ras/cAMP/PKA pathway, which leads to dephosphorylation of Ser\textsuperscript{598} and Ser\textsuperscript{620} of Msn2, thereby accumulating this transcription factor in the nucleus to activate the expression of \textit{GPX1}, (ii) Ca\textsuperscript{2+} activates Msn2 by yet unknown mechanism independent of the Ras/cAMP/PKA pathway, and (iii) Ca\textsuperscript{2+} activates Snf1 through three upstream protein kinases (Tos3, Elm1, and Sak1) to cause the early-stage-response of \textit{GPX1} (Fig. 8).
Physiological relevance to induction of GPX1 under glucose starved conditions

*GPX1*, *GPX2*, and *GPX3* were cloned as structural homologues of mammalian glutathione peroxidase (Inoue et al. 1999), although Gpx2 and Gpx3 have been identified as atypical 2-Cys peroxiredoxins that prefer to use thioredoxin as a reducing agent in vitro (Delaunay et al. 2002; Tanaka et al. 2005). The biochemical characterization of Gpx1 remains to be conducted, however, the enzyme is presumably an atypical 2-Cys peroxiredoxin as judged by the similarity of amino acid sequence with Gpx2 and Gpx3 (Tanaka et al. 2005). Of glutathione peroxidase homologues, as *GPX3* is constitutively expressed, a *gpx3*Δ mutant is sensitive to peroxides (Inoue et al. 1999). Meanwhile, a *gpx1*Δ mutant at log phase does not show any obvious phenotypes in terms of oxidative stress, which may be due to that the level of Gpx1 protein in a log phase of growth is basically quite low (Inoue et al. 1999), and therefore, the loss of Gpx1 may not give rise to a serious effect. We found that expression of *GPX1* is induced under glucose-starved conditions and in the diauxic shift, so, first, we compared the susceptibility to H2O2 between wild type and *gpx1*Δ cells under glucose-starved conditions. When cells pre-cultured in SD medium (2% glucose) were transferred to the glucose-starved medium containing 2 mM H2O2, cells were not able to grow. By contrast, when cells pre-cultured in the glucose-starved medium (0.1% glucose) were transferred to the fresh glucose-starved medium containing 2 mM H2O2, cells were able to grow, which is presumably due to the induction of antioxidant enzyme such as Gpx1 during adaptation period. As expected, the growth of a *gpx1*Δ mutant in the glucose-starved medium containing H2O2 was impaired (Fig. 7A). Since the expression of *GPX1* under glucose-starved conditions depends upon Msn2 and Msn4 as we have demonstrated in this study, cell growth of the *msn2Δmsn4Δ* mutant was also impaired.

On the other hand, we found that the Gpx1 level was kept high in the
post-diauxic shift (Fig. 5A), therefore, Gpx1 may have a role in the post-diauxic shift also. The mode of energy production of yeast changes from fermentation by glycolysis to respiration in mitochondria during diauxic shift (Werner-Washburne et al. 1993). It has been well known that functional mitochondria are necessary for assimilation of glycerol, and glycerol medium is sometimes used to mimic the conditions of post-diauxic shift. We then monitored the growth of yeast cells in the medium in which the amount of glucose is restricted. As a result, the growth of the \textit{gpx1}\textDelta mutant in the glycerol medium was lower by approximately 20% compared with that of wild type (Fig. 7B). Recently, Lee et al. have reported that Gpx3 protein physically interacts with dihydroxyacetone kinase 1 (Dak1), which is involved in glycerol metabolism (Lee et al. 2008); however, the role of Gpx3 in glycerol metabolism has not yet been elucidated. Since Gpx1 shows higher similarity with Gpx3 in amino acid sequence, Gpx1 may share the redundant function with Gpx3 in terms of the energy metabolism, especially in the assimilation of glycerol. As shown in Fig. 7B, the maximum growth of a \textit{gpx1}\textDelta\textit{gpx2}\textDelta\textit{gpx3}\textDelta mutant was further reduced in glycerol medium compared with that of the \textit{gpx1}\textDelta single mutant. Yeast glutathione peroxidase homologues may have some roles in the energy metabolism of non-fermentable carbon sources besides the function as an antioxidant enzyme.

The differences in the regulatory mechanism of the expression of three glutathione peroxidase homologues may reflect the distinct roles under different physiological conditions. Since \textit{GPX3} is constitutively expressed and the amount of Gpx3 protein is much higher compared with other two Gpxs, the deficiency in \textit{GPX3} increases the susceptibility to peroxides (Inoue et al. 1999). In addition, Gpx3 plays an important role in activation of Yap1, an oxidative-stress-responsive transcription factor (Delaunay et al. 2002; Okazaki et al. 2007). So, Gpx3 may be standing by for a sudden increase in the oxidative stress under normal growth conditions. On the other hand, the expression of \textit{GPX2} is induced under conditions of oxidative stress (Inoue et al. 1999;
Tsuzi et al. 2004a), and in the presence of CaCl₂ (Tsuzi et al. 2004b). It is known that Ca²⁺ increases the flux of electron in the electron transport chain in mitochondria, thereby accelerating the generation of reactive oxygen species (Nicotera et al. 1988; Pozniakovsky et al. 2005). We have previously reported that viability of a gpx2Δ mutant decreased when cells were treated with CaCl₂ and tert-butyl hydroperoxide simultaneously (Tanaka et al. 2005). Regarding the expression of GPX1, we have demonstrated in this study that the expression of GPX1 is induced when cells are starved of glucose or nitrogen. It has been reported that a limited supply of glucose increases oxygen respiration in yeast cells (Lin et al. 2002). Similarly, the reduction of TOR activity also enhances the oxygen consumption (Bonawitz et al. 2007). The increase in oxygen respiration accelerates the generation of reactive oxygen species in yeast cells (Sugiyama et al. 2000). In addition, oxidative stress occurs in cells of stationary phase of growth, in which medium nutrients are exhausted (Jakubowski et al. 2000; Herker et al. 2004; Favre et al. 2008). Hence, the complicated regulatory mechanism of the expression of GPXI (Ras/cAMP/PKA pathway, Snf1 pathway, and TOR pathway) may be necessary for yeast cells to adapt to the nutrient starvation-induced oxidative stress.

**Experimental procedures**

**Strains and media**

The *S. cerevisiae* strains used in this study are summarized in Table 1 in Supplementary material. Cells were cultured in YPD (2% glucose, 1% yeast extract, and 2% peptone), synthetic dextrose (SD) medium (2% glucose and 0.67% yeast nitrogen base without amino acid), or glycerol medium (3% glycerol, 0.05% glucose, and 0.67% yeast nitrogen base without amino acids) with appropriate amino acids and bases at 28°C. For glucose
starvation, cells were initially cultured in YPD or SD medium to a log phase of growth, collected by centrifugation, and suspended in a glucose-starved medium (0.1% glucose, 1% yeast extract and 2% peptone). For CaCl₂ treatment, 400 mM CaCl₂ was added to the medium. For nitrogen starvation, cells were initially cultured in SD medium to a log phase of growth, collected by centrifugation, and suspended in a nitrogen-starved medium (2% glucose, and 0.17% yeast nitrogen base without amino acids and ammonium sulfate) with appropriate amino acids and bases at 28°C. As a control, cells were suspended in a nitrogen-sufficient medium (nitrogen-starved medium plus 0.5% ammonium sulfate) with appropriate amino acids and bases.

**Gene disruption**

The RAS2 gene was amplified by PCR using primers RAS2-F and RAS2-R. The products were digested with BamHI and HindIII, and cloned into the BamHI-HindIII site of pUC19. The resultant plasmid (pUC19-RAS2) was digested with PstI and Ball, and the fragment containing a part of and the open reading frame (ORF) of RAS2 was replaced with TRP1. This plasmid (pUC19-ras2Δ::TRP1) was digested with BamHI and HindIII, and the BamHI/HindIII fragment containing a ras2Δ::TPR1 cassette was introduced into *S. cerevisiae* to disrupt RAS2.

The GIS1 gene was amplified by PCR using GIS1-F and GIS1-R. SacI and SphI sites were designed in GIS1-F and GIS1-R, respectively (underlined). The PCR products were digested with SacI and SphI, and cloned into the SacI-SphI site of pUC19. The resultant plasmid (pUC19-GIS1) was digested with NsiI, and the fragment containing a part of ORF of GIS1 was replaced by LEU2. This plasmid (pUC19-gis1Δ::LEU2) was digested with SacI and SphI, and the SacI/SphI fragment containing a gis1Δ::LEU2 cassette was introduced into *S. cerevisiae* to disrupt GIS1.

To construct a msn4Δ::TPR1 mutant, pUCmsn4 (Inoue *et al.* 1998) was
digested with EcoRV, and the EcoRV fragment was replaced by TRP1. The resulting plasmid (pUC-msn4Δ::TRP1) was digested with NruI and AccI, and the NruI/AccI fragment containing a msn4Δ::TRP1 cassette was introduced into S. cerevisiae.

To construct gpx1Δ::HIS3, gpx3Δ::LEU2, msn2Δ::HIS3, snf1Δ::HIS3, hog1Δ::LEU2, cnb1Δ::HIS3, crz1Δ::LEU2, and mig1Δ::HIS3 mutants, each mutated allele was amplified by PCR using the following primer sets: GPX1-F and GPX1-R, GPX3-F and GPX3-R, MSN2ΔHIS3F and MSN2ΔHIS3R, SNF1-F and SNF1-R, HOG1-F and HOG1-R, CNB1FhincII and CNB1RhincII, CRZ1-S and CRZ1-R, or MIG1-F and MIG1-R, and the genomic DNA of the respective mutants constructed in our previous studies (Inoue et al. 1999; Tsujimoto et al. 2000; Tsuzi et al. 2004a, 2004b; Maeta et al. 2005). To construct gpx2Δ::kanMX4, msn2Δ::kanMX4, msn4Δ::kanMX4, mig2Δ::kanMX4, ssn6Δ::kanMX4 and tup1Δ::kanMX4 mutants in the YPH250 background, genomic DNA of each mutant in the BY4741 background (Invitorogen) was used as a template for PCR with the following primer sets: GPX2-F and GPX2-R, MSN2ΔHIS3F and MSN2ΔHIS3R, MSN4-F and MSN4-R, MIG2-F and MIG2-R, SSN6-F and SSN6-R, and TUP1-F and TUP1-R. Each PCR fragment, which contains the disrupted allele of each gene replaced by kanMX4, was introduced into YPH250. Disruption of each gene was confirmed by PCR.

All PCR primers used in this study are summarized in Table 2 in Supplementary material.

Construction of GPX1-lacZ reporter gene

To amplify the region between -365 and +8 of GPX1, taking the translation initiation codon (ATG) as +1, PCR was done with GPX1-lacZ-F and GPX1-lacZ-R. SalI and EcoRI sites were designed in GPX1-lacZ-F and GPX1-lacZ-R, respectively (underlined). The PCR fragment was digested with SalI and EcoRI, and cloned into the SalI-EcoRI site of
YIp358R. The resultant plasmid (YIp358R-GPX1-lacZ) was digested with NcoI, and the linearized fragment was integrated into the *URA3* locus of YPH250.

To construct the *GPX1*-*lacZ* gene carrying the mutated STRE (STRE1mut and STRE2mut), an overlap extension PCR was conducted (Ho *et al.* 1989). GPX1-*lacZ*-F and GPX1-*lacZ*-R were used as the external primers. For the site-directed mutagenesis, four internal primers (STRE1mut-F, STRE1mut-R, STRE2mut-F and STRE2mut-R) were designed to change the consensus sequence of STRE (5′-CCCCT-3′) to 5′-CATCT-3′ (Zahringer *et al.* 2000), where the mutated sequence is underlined. To create the mutation in STRE1 (-303 to -298), the first PCR was performed with the following primer sets: GPX1-*lacZ*-F plus STRE1mut-R, and GPX1-*lacZ*-R plus STRE1mut-F. The PCR product was digested with *SalI* and *EcoRI*, and the resultant fragment was inserted into the *SalI*-*EcoRI* site of YIp358R to yield YIp358R-STRE1mut-GPX1-*lacZ*. To create the mutation in STRE2 (-248 to -243), the first PCR was performed with the following primers: GPX1-*lacZ*-F plus STRE2mut-R and GPX1-*lacZ*-R plus STRE2mut-F. The second PCR was done with GPX1-*lacZ*-F and GPX1-*lacZ*-R as the primers, and the mixture of the PCR products of each first PCR as the template. The PCR product was cloned into YIp358R as described above (YIp358R-STRE2mut-GPX1-*lacZ*). To construct the *GPX1*-*lacZ* gene carrying both STRE1mut and STRE2mut (STRE1/2mut), the first PCR was performed with the following primer sets: GPX1-*lacZ*-F plus STRE2mut-R, and GPX1-*lacZ*-R plus STRE2mut-F using YIp358R-STRE1mut-GPX1-*lacZ* as a template. The second PCR was done using GPX1-*lacZ*-F and GPX1-*lacZ*-R, and the mixture of the PCR products of each first PCR as the template. The PCR product was digested with *SalI* and *EcoRI*, and the resultant fragment was inserted into the *SalI*-*EcoRI* site of YIp358R to yield YIp358R-STRE1/2mut-GPX1-*lacZ*. Introduction of the mutation was verified by DNA sequencing.
Construction of *GPX1-lacZ* promoter fusion plasmid with pTBA30

The promoter region of *GPX1* was amplified with the primers, GPX-pTBA-2F and GPX1-pTBA-2R (-345 to -182). The PCR product was digested with *Xho*I, and inserted into the *Xho*I site of pTBA30, which contains the TATA box of *CYC1* followed by *lacZ* (Galius-Duerner *et al.* 1997). The resultant plasmid containing the region from -345 to -182 was named pGPX1-CYC1-lacZ. The insertion of the fragment in the correct orientation was verified by DNA sequencing.

Construction of *GPX1-3HA*

To add the HA-tag at the C terminus of Gpx1, PCR primers were designed (GPX1-HA-F and GPX1-HA-R) to amplify the region between the 5’-flanking region of *GPX1* and the triplet before the translation termination codon of *GPX1*. The *Xho*I and *Bgl*II sites were designed in GPX1-HA-F and GPX1-HA-R, respectively (underlined). The PCR fragment was digested with *Xho*I and *Bgl*II, and the resultant fragment was inserted into the *Xho*I-*Bgl*II site of the plasmid pSLF172 (Forsburg & Sherman 1997). The resultant plasmid (pSLF172-GPX1) was digested with *Hind*III and *Sal*I, and the DNA fragment containing *GPX1-3HA* was cloned into the *Hind*III-*Sal*I site of YIp5. The resultant plasmid (YIp5-GPX1-3HA) was linearlized with *Nco*I, and integrated into the *URA3* locus of *S. cerevisiae*.

β-Galactosidase assay

Cells carrying a genome-integrated *GPX1-lacZ* reporter gene were cultured in YPD medium until an appropriate phase of growth, and disrupted with glass beads in 10 mM potassium phosphate buffer (pH 7.0). To culture the cells carrying the plasmid (pTBA30)-borne *GPX1-lacZ*, cells were grown in SD medium with appropriate amino
acids and bases until the mid-log phase. β-Galactosidase activity was measured as described previously (Inoue et al. 1998). One unit of β-galactosidase activity was defined as the amount of enzyme that increases the $A_{420}$ by 1000 per h at 30°C. The protein concentration was determined by the method of Bradford (1976).

**Western blotting**

Cells carrying GPX1-3HA were cultured in YPD medium, and disrupted with glass beads in 10 mM Tris-HCl buffer (pH 7.0) containing 1mM phenylmethylesulfonyl fluoride. Cell homogenate was centrifuged at 14000 rpm for 10 min at 4°C, and the supernatant (cell extract) was subjected to SDS-PAGE. The separated proteins were transferred to a PVDF membrane (Immobilon; Millipore). Anti-HA monoclonal antibody (6E2, Cell Signaling) was used to detect Gpx1-3HA. To detect Cdc28 as a loading control, the membrane was treated in 0.2 M glycine (pH 2) for 5 min, and reprobed with anti-Cdc2 antibody (Santa Cruz Biotechnology). Anti-mouse IgG or anti-rabbit IgG conjugated with alkaline phosphatase (Cell Signaling) or horseradish peroxidase was used as a secondary antibody. Immunoreacting bands were visualized with a BCIP-NBT kit (Nacalai tesque).

**Northern blotting**

Total RNA was prepared by the method of Schmitt et al. (1990), and 20 µg of RNA was loaded onto a denaturing agarose gel. To prepare $^{32}$P-labeled DNA probes, GPX1 was amplified using primers GPX1-GFP-F2 and GPX1-GFP-R, and the PCR fragment was labeled with [α-$^{32}$P]dCTP (Amersham) using a kit (Takara).

**Phosphorylation of Msn2**

To determine the phosphorylation state of Msn2 at Ser$^{582}$ and Ser$^{620}$, cells carrying
Msn2-GFP (Görner et al. 1998) were cultured in SD medium until a log phase of growth, and starved of glucose or treated with 400 mM CaCl₂ at 28 °C. Cells were collected at a prescribed time, and disrupted with glass beads in 100 mM Tris-HCl buffer (pH 7.0) containing 5 mM MgCl₂, 10% glycerol, 0.1% Triton X-100, 50 mM NaCl, 1 mM dithiothreitol, and protease inhibitor cocktail (Nakalai tesque). Cell extracts (20 µg proteins) were subjected to SDS-PAGE. Anti-GFP antibody was used to detect Msn2-GFP. Anti-phospho-Ser⁵⁸² of Msn2 antibody, kindly provided by Dr. C. Brocard (De Wever et al. 2005), was used to determine the phosphorylation state of Ser⁵⁸². Anti-phospho-CREB (phospho-Ser¹³³) antibody (Cell Signaling) was used to determine the phosphorylation state of Ser⁶²⁰ (Görner et al. 2002).

**Phosphorylation of Snf1**

Cells were cultured in YPD medium until a log phase of growth, and then starved of glucose or treated with 400 mM CaCl₂ at 28°C for a prescribed time. To determine the phosphorylation state of Thr²¹⁰ of Snf1, cellular proteins were prepared essentially as described by Bell et al. (2001). After preparation of the samples for SDS-PAGE, the protein concentration was determined with a RC DC Protein Assay kit (BIORAD). Anti-phospho-Thr¹⁷² of mammalian AMPK polyclonal antibody (Cell Signaling) was used to determine the phosphorylation state of Thr²¹⁰ of Snf1 (Momcilovic et al. 2006). To detect Snf1 protein, anti-poly-His monoclonal antibody (HIS-1, Sigma) was used (Momcilovic et al. 2006).

**Measurement of glucose in the medium**

Cells were cultured in 500-ml Sakaguchi flasks containing 300 ml of YPD medium, and 1 ml of culture was withdrawn periodically. After the removal of cells by centrifugation, the glucose concentration in the supernatant was measured using a kit (Glucose test Wako).
C, Wako).

**Fluorescence microscopy**

The localization of Msn2 was determined using a GFP-tagged Msn2 as we described previously (Tsuzi *et al.* 2004b). The localization of Snf1 was examined by indirect immunofluorescence microscopy using anti-poly-His monoclonal antibody.

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**References**


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Figure legends

Figure 1 Expression of GPX1 induced by glucose starvation and CaCl2. (A) Cells carrying a GPX1-lacZ reporter gene were cultured in YPD medium until $A_{610}=0.8$, and then treated with 400 mM CaCl2 or transferred to fresh medium containing 0.1% glucose (Low Glc) for 90 min, and β-galactosidase activity was measured. In control (Cont.) experiments, cells were transferred to fresh YPD medium. Values are the average of three independent experiments ± standard deviation. For Western blotting, cells carrying GPX1-3HA were cultured in YPD medium until $A_{610}=0.8$, and treated with CaCl2 or
starved of glucose for 90 min. Gpx1 protein levels were then determined using anti-HA monoclonal antibody. Cdc28 is a loading control of Western blotting. For Northern blotting, cells were cultured in YPD medium until $A_{610}=0.8$, and treated with CaCl₂ or starved of glucose for 30 min. Total RNA was then prepared. (B) Each mutant carrying the GPX1-lacZ reporter gene was cultured in YPD medium until $A_{610}=0.8$, and treated with 400 mM CaCl₂ for 90 min, then β-galactosidase activity was measured. Values are the average for three independent experiments ± standard deviation.

**Figure 2** Effect of disruption of MSN2 and MSN4 on GPX1 expression. (A) Configuration of the GPX1 promoter. (B) Cells of the wild-type (WT) or msn2Δmsn4Δ cultured in YPD medium until $A_{610}=0.8$ were treated as described in the legend of FIG. 1, and Northern blotting were done. (C) Wild-type cells carrying each mutant STRE-driven GPX1-lacZ were cultured in YPD medium until $A_{610}=0.8$, and treated as described in the legend of FIG. 1. The values of β-galactosidase activity are the average for three independent experiments ± standard deviation. Slot 1, control; slot 2, low glucose; and slot 3, CaCl₂. (D) Cells of the wild type or an msn2Δ::HIS3 msn4Δ::TRP1 mutant carrying the GPX1 promoter fusion plasmid (pGPX1-CYC1-lacZ) were cultured in SD medium until $A_{610}=0.8$. After being collected by centrifugation, cells were suspended in 0.1% glucose+YP medium (Low Glc) or YPD medium containing 400 mM CaCl₂. Cells were culture for another 90 min, and β-galactosidase activity was measured. Values are the average for three independent experiments ± standard deviation.

**Figure 3** (A) Induction of GPX1 expression in mutants defective in glucose signaling. Cells of the wild type (WT) and ras2Δ mutant carrying GPX1-3HA were grown in YPD medium until $A_{610}=0.8$, and treated with 400 mM CaCl₂ or starved of glucose (Low Glc) for 30 min. Gpx1 protein levels were then determined. In control (Cont.) experiments,
cells were transferred to fresh YPD medium. (B) Ca\(^{2+}\) signal converges on Msn2/Msn4. Cells (wild type, \(ras2\Delta\), \(msn2\Delta msn4\Delta\), and \(msn2\Delta msn4\Delta ras2\Delta\)) carrying a \(GPX1\)-lacZ reporter gene were cultured in YPD medium until \(A_{610}=0.8\), and then treated with 400 mM CaCl\(_2\) for 90 min, and \(\beta\)-galactosidase activity was measured. In control experiments, cells were transferred to fresh YPD medium. Values are the average of three independent experiments \(\pm\) standard deviation. (C) Localization of Msn2. Cells of wild type or \(ras2\Delta\) mutant carrying GFP-tagged Msn2 were cultured in SD medium until \(A_{610}=0.3\), treated as described in (A) for 10 min, and then localization of Msn2-GFP was monitored. (D) Phosphorylation of Ser\(^{582}\) and Ser\(^{620}\) of Msn2. Cells of the wild type carrying the Msn2-GFP plasmid were cultured in SD medium until \(A_{610}=0.8\), and treated as described in (A) for 30 or 60 min. The phosphorylation state of each serine residue in Msn2 was determined using respective specific antibodies. The Msn2-GFP protein level was verified using anti-GFP antibody.

**Figure 4** Role of Snf1 for expression of \(GPX1\) in response to glucose starvation and Ca\(^{2+}\). (A) Cells of the wild type (WT) and \(snf1\Delta\) mutant carrying \(GPX1\)-3HA were grown in YPD medium until \(A_{610}=0.8\), and treated with 400 mM CaCl\(_2\) or starved of glucose (Low Glc) for the prescribed time as indicated in the figure, and Gpx1 protein levels were determined by Western blotting. (B) Cells of the wild type or a \(tos3\Delta sak1\Delta elm1\Delta\) mutant were cultured in YPD medium until \(A_{610}=0.5\), and treated as described in (A). Cells were collected at the prescribed time, and the phosphorylation of Thr\(^{210}\) of Snf1 was monitored. (C) Cells of wild type or a \(cnb1\Delta\) were cultured in YPD medium until \(A_{610}=0.5\), treated as described in (A) for 30 min, and the phosphorylation of Thr\(^{210}\) of Snf1 was monitored. (D) Localization of Snf1 was monitored by indirect immunofluorescence microscopy using anti-poly-His monoclonal antibody. Since no signal was detected in \(snf1\Delta\) cells, this monoclonal antibody is valid to detect Snf1. DAPI (4',6-diamino-2-phenylindole) was
used to stain the nucleus.

**Figure 5** Change of Gpx1 level during growth. (A) Cells carrying *GPX1-3HA* were cultured in YPD medium, and cell growth was monitored periodically (*squares*). At each point, glucose concentration in the medium (*closed circles*) were also determined. Gpx1 protein level was determined by Western blotting at the time indicated in the figure. (B) Cells of *msn2Δmsn4Δ* and *msn2Δmsn4Δgis1Δ* were cultured in YPD medium, and the Gpx1 protein level was determined by Western blotting at the time indicated in the figure.

**Figure 6** Effect of nitrogen starvation and TOR pathway on expression of *GPX1*. (A) Cells carrying *GPX1-3HA* were cultured in YPD medium until *A*$_{610}$=0.8, transferred to nitrogen-starved medium or nitrogen-sufficient medium, and further cultured for the period as indicated in the figure to monitor the levels of Gpx1 protein. The glucose concentration in the medium is shown below each panel. Cdc28 is a loading control of Western blotting. (B) Wild-type cells were cultured in YPD medium until *A*$_{610}$=0.8 and treated with 200 ng/ml rapamycin for the period indicated in the figure, and total RNA was prepared.

**Figure 7** Physiological relevance to induction of *GPX1* under glucose starved conditions. (A) Cells of wild type (WT), *gpx1Δ*, and *msn2Δmsn4Δ* were cultured in low glucose (0.1%) medium for 16 h, and an aliquot was transferred to the fresh low glucose medium containing 2 mM H$_2$O$_2$. The data indicate *A*$_{610}$ of culture for 28 h. (B) Cells of wild type (WT), *gpx1Δ*, and *gpx1Δgpx2Δgpx3Δ* (*gpx1/2/3Δ*) were cultured in glycerol medium, and *A*$_{610}$ was monitored periodically.

**Figure 8** A schematic diagram of the regulation of *GPX1* expression. (*Left panel*) Ca$^{2+}$
induces the phosphorylation of Snf1 at Thr$^{210}$, leading to the expression of $GPX1$ in an early-stage response. However, the Ca$^{2+}$-induced expression of $GPX1$ was observed in $snf1\Delta$ cells, because Msn2 is activated following treatment with CaCl$_2$. Dephosphorylation of Ser$^{582}$ and Ser$^{620}$ of Msn2 in response to Ca$^{2+}$ might be achieved through an inhibition of the Ras/cAMP/PKA pathway. In addition, Ca$^{2+}$ signal seems sent to Msn2/Msn4 independent of the Ras/cAMP/PKA pathway. (Right panel) Glucose starvation causes the activation of Snf1 via phosphorylation of Thr$^{210}$ by three upstream protein kinases. Meanwhile, glucose starvation deactivates the Ras/cAMP/PKA pathway, which leads to dephosphorylation of Ser$^{582}$ and Ser$^{620}$ of Msn2, and subsequently nuclear Msn2 activates transcription of $GPX1$. The details for the model are given in the text.