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Distinct regulatory mechanism of yeast *GPX2* encoding phospholipid hydroperoxide glutathione peroxidase by oxidative stress and a calcineurin/Crz1-mediated Ca^{2+} signaling pathway

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Abstract The *GPX2* gene encodes a homologue of mammalian phospholipid hydroperoxide glutathione peroxidase in *Saccharomyces cerevisiae*. Previously, we have reported that the oxidative stress-induced expression of *GPX2* is strictly regulated by Yap1 and Skn7 transcription factors. Here, we found that the expression of *GPX2* is induced by $CaCl_2$ in a calcineurin (CN)/Crz1-dependent manner, and the CN-dependent response element was specified in the *GPX2* promoter. Neither Yap1 nor Skn7 was required for Ca^{2+}-dependent induction of *GPX2*, therefore, distinct regulation for the oxidative stress response and Ca^{2+} signal response for *GPX2* exists in yeast cells.

Keywords: Glutathione peroxidase; Calcineurin; Crz1; Yeast; Ca^{2+} signaling

1. Introduction

All aerobic organisms use molecular oxygen for respiration or oxidation of nutrients to acquire energy efficiently and subsequently molecular oxygen is reduced to H_2O through acceptance of four electrons. During the reduction of molecular oxygen, several reactive oxygen species are formed. To protect cellular components from oxidative damage, organisms have evolved the antioxidant system [1]. Glutathione peroxidase (GPx) is one of the important antioxidant enzymes. GPx catalyzes the reduction of H_2O_2 and lipid hydroperoxides to H_2O and corresponding alcohols using glutathione as a reducing power [2,3].

Previously, we have reported that the budding yeast *Saccharomyces cerevisiae* has three glutathione peroxidase homologues (*GPX1*, *GPX2* and *GPX3*) [2]. We have also found that only *GPX2* responds to oxidative stress [2], and both Yap1 and Skn7 transcription factors are necessary for oxidative stress-induced expression of this gene [4]. We identified the *cis*-acting elements for Yap1 and Skn7 within the *GPX2* promoter [4]. Besides such elements, the *GPX2* promoter possesses the sequence of which is completely consistent with the motif termed STRE (stress response element, 5′-CCCCT-3′) to which Msn2 transcription factor binds. A gene carrying the STRE usually responds to a wide variety of stress stimuli such as heat shock stress, osmotic stress and oxidative stress [5], however, we revealed that Msn2 was not involved in the oxidative stress response of *GPX2* [4]. To gain further insights into the regulatory mechanism of *GPX2* expression, we analyzed responsiveness of *GPX2* to various environmental stimuli. Intriguingly, in this study we found that the expression of *GPX2* is induced by Ca^{2+}. We clarify that the Ca^{2+}-dependent expression of *GPX2* is regulated by the calcineurin (CN)/Crz1 pathway, which is independent of the Yap1/Skn7-mediated regulation. We also specify the *cis*-acting element responsible for the Ca^{2+} response within the *GPX2* promoter. As far as we know, this is the first report demonstrating that CN regulates the expression of a gene encoding GPx.

2. Materials and methods

2.1. Strains and media

All yeast strains of *S. cerevisiae* used in this study have the YPH250 background (*MAT***a** *trp1*-Δ*1 his3*-Δ*200 leu2*-Δ*1 lys2*-801 *ade2*-101 *ura3*-52). Cells were cultured in YPD (2% glucose, 1% yeast extract and 2% peptone) or SD minimal medium (2% glucose and 0.67% yeast nitrogen base without amino acids) with appropriate amino acids and bases at 28 °C with reciprocal shaking.

2.2. Gene disruption

The *CRZ1* gene was amplified by PCR using the following primers: CRZ1-S, 5′-TAATATAGTGCAGCATGCAACTTGC-3′ and CRZ1-R, 5′-CACGTAAAACGGATCCTCATACAATA-3′. The *Sph*I and *Bam*HI sites were designed (underlined) for CRZ1-S and CRZ1-R, respectively. The PCR product was digested with *Sph*I and *Bam*HI and cloned into the *Sph*I–*Bam*HI site of pUC19. The resultant plasmid (pCR1) was digested with *Pst*I and *Xho*I, and a part of the open reading frame of *CRZ1* was replaced with the *LEU2* gene. The resultant plasmid (pCR1ΔLeu2) was digested with *Sph*I and *Bam*HI, and the DNA fragment containing the *crz1*Δ::*LEU2* cassette was introduced to *S. cerevisiae*. Electroporation method using the Gene Pulser II (Bio-Rad) was adopted for yeast transformation under conditions of 1.5 kV, 200 Ω, and 25 μF.

A *cnb1*::*HIS3* allele of a mutant in the W303-1A background, which was donated by Dr. T. Miyakawa [6], was amplified by PCR with the following primers: CNB1FhincII, 5′-CATGTGGCAAGAACAGCGGGATGTATAGGT-3′ and CNB1RhincII, 5′-ATTACTGAAGGATGCGAGGTTCGAACTCGC-3′. The *CNB1* gene of *S. cerevisiae* YPH250 was disrupted using this DNA fragment.

Construction of *msn2*Δ::*HIS3*, *msn4*Δ::*ADE2*, *yap1*Δ::*HIS3*, *skn7*Δ::*TRP1* and *gpx3*Δ::*LEU2* was described previously [2,4,7].

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2.3. Construction of GPX2-lacZ reporter gene

Construction of the *GPX2-lacZ* fusion plasmid was carried out essentially as described previously [4]. The *GPX2-lacZ*-1 plasmid (−709/+7) contains a 709-bp 5′-non-coding region of *GPX2* fused with the sequence encoding the first 2 amino acids of Gpx2. A series of promoter-deleted *GPX2-lacZ* plasmids [*GPX2-lacZ*-2 (−485/+7), *GPX2-lacZ*-3 (−306/+7), *GPX2-lacZ*-3a (−264/+7), *GPX2-lacZ*-4 (−204/+7) and *GPX2-lacZ*-5 (−133/+7)] were constructed using the forward (GPX2-2, GPX2-3, GPX2-3a, GPX2-4 and GPX2-5) and reverse (GPX2lacR2) primers as follows: GPX2-1, 5′-TTACCGTTGTCGACCTTGCTCTAC-3′; GPX2-2, 5′-TCTGTGTTTGTCGACGTAACATA-3′; GPX2-3, 5′-GCAATTTTACTGTCGACTGTTTA-3′; GPX2-3a, 5′-CTCGGCCGGCCATGTCGACACAATTAGTAA-3′; GPX2-4, 5′-CCACACATGTCGACAAAGGCATTA-3′; GPX2-5, 5′-GCTTTAAAAAATGTCGACGTACTTTTGTTA-3′; GPX2lacR2, 5′-TCATAAAGAATTCTGGTCATTTTGAATTAT-3′. Each forward and reverse primer was designed to contain *Sal*I (forward) and *Eco*RI (reverse) sites, respectively (underlined). The DNA fragments amplified by PCR were digested with *Sal*I and *Eco*RI, and cloned into the *Sal*I–*Eco*RI site of YIp358R. The resultant *GPX2-lacZ* plasmids were digested with *Nco*I and integrated into the *URA3* locus of YPH250.

2.4. Enzyme assay

Cells were cultured in a 200-ml flask containing 50 ml each of YPD medium at 28 °C. When the A_{610} reached approximately 1.0, 0.4 mM H_2O_2, 200 mM $CaCl_2$ and/or 1 μg/ml FK506 (Fujisawa Pharmaceutical Co., Ltd., Tokyo, Japan) were added and the cells were cultured for another 1 h at 28 °C. Preparation of cell extracts and assay of β-galactosidase activity and catalase activity were done as described previously [7]. One unit of activity was defined as the amount of enzyme that increases the A_{420} by 1000 per hour at 30 °C. Protein concentration was determined by the method of Bradford [8].

2.5. Northern blotting

Northern blot analysis of *GPX2* was done as described previously [2,4]. Briefly, total RNA was prepared from the cells treated with 200 mM $CaCl_2$ in the presence or absence of 1 μg/ml FK506 for 30 min in YPD medium. To prepare the probe, PCR was performed to amplify the coding region of *GPX2* with the following primer set: 5′-TAAAAGCTTATGACCACATCTTTTTATGAT-3′ and 5′-CAAAGGATCCTTTTACTTAACAGGCTTTGG-3′. The PCR fragment was labeled with [α-^{32}P] dCTP.

2.6. Electrophoretic mobility shift analysis (EMSA)

An oligonucleotide probe containing the target site was generated by PCR amplification. To amplify the CDRE-like region (−190/−118), GPX2-crz1-S (5′-CCATCGATCATTGTCTCCGTATTAGTGCA-3′) and GPX2-crz1-AS (5′-GACTAGTACAATAACAAAAGTACCTGCACA-3′) were used. The *Cla*I and *Spe*I sites (underlined) were designed in the forward primer (GPX2-crz1-S) and reverse primer (GPX2-crz1-AS), respectively. The amplicon was digested with *Cla*I and *Spe*I, and the fragment was cloned into the *Cla*I–*Spe*I site of pRS416. The resultant plasmid (pRS416-CDRE) was digested with *Cla*I and *Spe*I, and the 3′-end of each DNA fragment was labeled by Klenow with [α-^{32}P] dCTP. The ^{32}P labeled probe was purified by a Sephadex G-50 spin column.

The DNA binding reactions were carried out as described previously [4]. Briefly, the reaction mixture contained 25 mM Tris–HCl buffer (pH 7.5), 50 mM NaCl, 2 mM EDTA, 7 mM $MgCl_2$, 10% glycerol, cell extracts (20 μg protein), 2 ng of ^{32}P 3′-end labeled probes and 1 μg of poly(dI-dC) in a total volume of 20 μl. The mixture was kept for 15 min at room temperature and then for another 15 min on ice. After electrophoresis on a non-denaturing polyacrylamide gel (4%), the gel was dried onto Whatman 3MM paper followed by autoradiography with a Fujix Bio-Imaging Analyzer BAS2000 (Fuji Photo Film, Tokyo, Japan).

2.7. Preparation of cell extracts containing GFP-Crz1 for EMSA

A CEN-type GFP-Crz1 plasmid (pAMS463) [9], which was donated by Dr. M. Cyert, was introduced into a *crz1*Δ mutant and the resultant transformant was cultured in SD minimal medium until the $A_{610} = 1.0$. Cells were disrupted with glass beads in 200 mM Tris–HCl buffer (pH 8.0) containing 10 mM $MgCl_2$, 10% glycerol and a protease inhibitor cocktail for yeast (Sigma–Aldrich Co., St. Louis, MO), and the cell extracts were used for electrophoretic mobility shift analysis (EMSA).

2.8. Fluorescence microscopy

Cells carrying the Msn2-GFP plasmid (CEN-type) [10], which was donated by Dr. C. Schüller, and the GFP-Crz1 plasmid [9] were cultured in SD minimal medium with appropriate amino acids and bases until the $A_{610} = 0.5$, and localization of GFP-tagged proteins was determined using fluorescent microscopy (Olympus BX51). Msn2-GFP and GFP-Crz1 have been reported to be able to complement deficiency of *MSN2* and *CRZ1*, respectively [9,10].

3. Results

3.1. Expression of GPX2 is induced by Ca^{2+}

We have previously reported that Msn2 and Msn4 are not necessary for the oxidative stress response of *GPX2* even though the *GPX2* promoter contains the STRE [4]. It has been reported that the expression of many genes possessing the STRE is induced by several different stress stimuli such as heat shock and osmotic stress [5]. However, the expression of *GPX2* was not induced by hyper- and hypo-osmotic stress, heat shock stress, or ethanol stress (Fig. 1A). Nevertheless, interestingly, we found that *GPX2-lacZ* was induced by 200–300 mM $CaCl_2$ (Fig. 1B). The increase in the level of *GPX2* expression was confirmed by Northern blotting (Fig. 3B).

We suspected that Msn2 and/or Msn4 might be involved in the Ca^{2+}-induced expression of *GPX2*, because the Msn2–GFP fusion protein was concentrated in the nucleus when the cells were treated with 200 mM $CaCl_2$ (Fig. 2A). This phenomenon has not yet been reported so far. However, the expression of *GPX2-lacZ* was still induced by $CaCl_2$ in *msn2*Δ, *msn4*Δ (data not shown) and *msn2*Δ*msn4*Δ mutants (Fig. 2B). Therefore, neither Msn2 nor Msn4 is involved in the Ca^{2+} signal response of *GPX2*, although, since it has been reported that Msn2 participates in the expression of various genes, Ca^{2+}-dependent activation of Msn2 may have some physiological function to regulate them. For example, *CTT1*, coding for cytosolic catalase, contains several STREs within its promoter [11,12], and we found that expression of *CTT1-lacZ* [13] (untreated, 0.67 ± 0.09 unit/mg protein; Ca^{2+}-treated, 42.1 ± 3.5 unit/mg protein) and catalase activity (untreated, 0.34 ± 0.09 unit/mg protein; Ca^{2+}-treated, 7.17 ± 0.98 unit/mg protein) increased following Ca^{2+} treatment. Regulatory mechanism of *CTT1* expression in response to Ca^{2+} will be discussed elsewhere.

Next, we investigated whether Yap1 and/or Skn7 are involved in this event. As shown in Fig. 2B, Ca^{2+}-induced expression of *GPX2* occurred in the mutants defective in *YAP1* and *SKN7*. Delaunay et al. [14] reported that the *GPX3* gene product functions as a redox modulator for Yap1 activity. To determine whether Gpx3 is involved in the Ca^{2+} signaling process of *GPX2*, the *GPX2-lacZ* reporter gene was introduced to a *gpx3*Δ mutant. As shown in Fig. 2B, no induction was observed with H_2O_2 treatment, although the Ca^{2+}-induced expression of *GPX2-lacZ* was normal in *gpx3*Δ cells. Therefore, the expression of *GPX2* by $CaCl_2$ is independent of the Yap1/Skn7-mediated pathway.

3.2. Calcineurin regulates the GPX2 gene expression

Calcineurin is a Ca^{2+}/calmodulin-dependent serine/threonine protein phosphatase that is highly conserved in eukaryotes from yeasts to mammals and plays a crucial role in the Ca^{2+} signaling pathway [15,16]. Crz1 is assumed to be the only transcription factor that functions under the control of CN

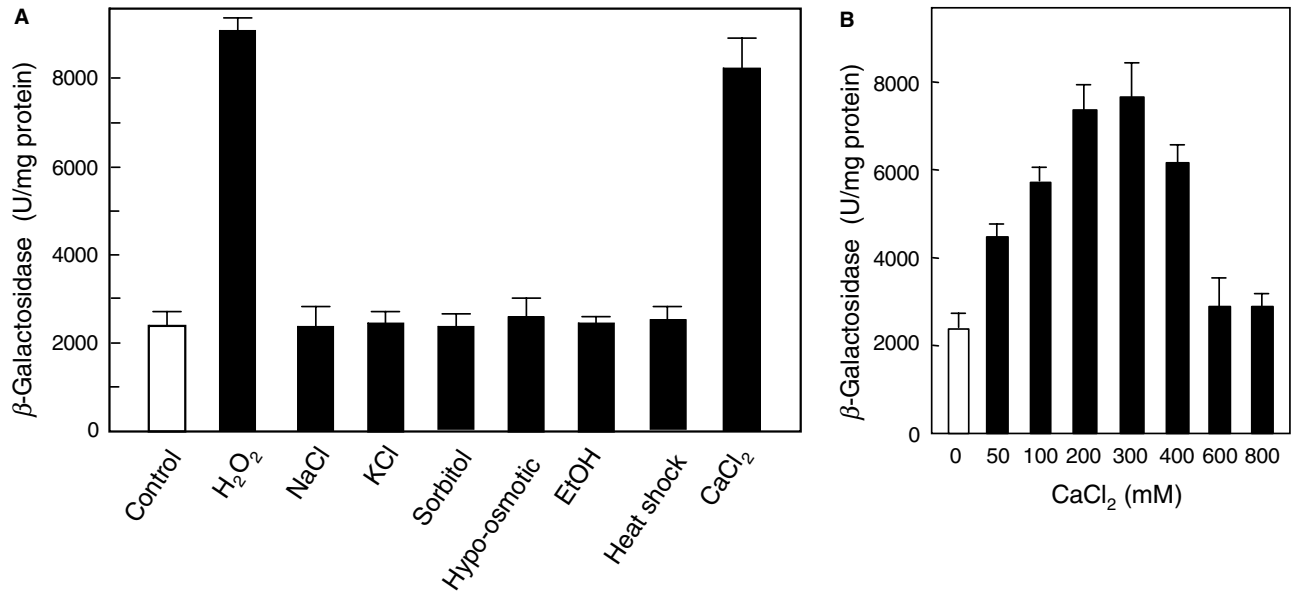

Fig. 1. Expression of *GPX2* is induced by Ca^{2+}. (A) Cells carrying *GPX2-lacZ*-1 were cultured in YPD medium until the exponential phase, and treated with 0.4 mM H_2O_2, 0.5 M NaCl, 0.5 M KCl, 1 M sorbitol, 7.5% ethanol (EtOH) or 200 mM $CaCl_2$ for 1 h. For heat shock treatment, cells cultured at 28 °C were transferred to 37 °C and incubation was continued for another 1 h. For hypo-osmotic stress, cells cultured in YPD medium containing 1 M sorbitol were collected by centrifugation, suspended in YPD medium and incubated for 1 h. After each stress treatment, the expression of *GPX2* was quantified by measuring β-galactosidase activity. Data are averages ± S.D. of three independent experiments. (B) Cells carrying *GPX2-lacZ*-1 were cultured in YPD medium until the exponential phase and various concentrations of $CaCl_2$ were added. After 1 h, β-galactosidase activity was assayed. Data are averages ± S.D. of three independent experiments.

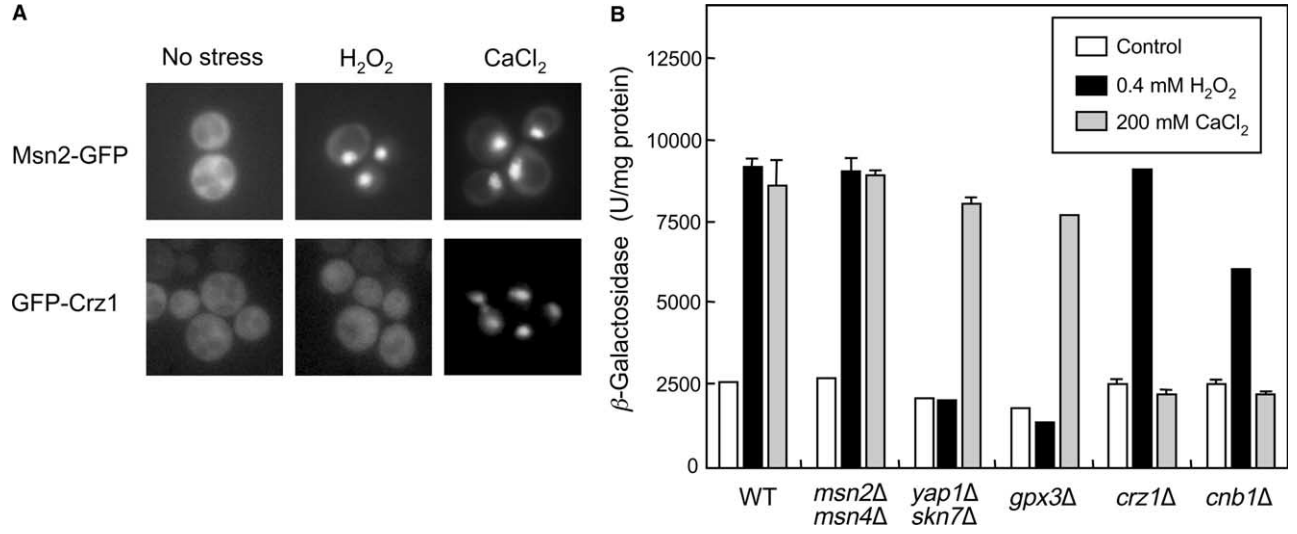

Fig. 2. Effects of $CaCl_2$ and H_2O_2 on the expression of *GPX2*. (A) *msn2*Δ cells carrying the Msn2-GFP plasmid (upper panels) or *crz1*Δ cells carrying the GFP-Crz1 plasmid (lower panels) were cultured in SD minimal medium until the $A_{610} = 0.5$, and 0.4 mM H_2O_2 or 200 mM $CaCl_2$ was added. After 30 min, localization of Msn2-GFP and GFP-Crz1 was monitored. (B) Cells carrying *GPX2-lacZ*-1 in different genetic backgrounds were cultured in YPD medium until the exponential phase, and 0.4 mM H_2O_2 or 200 mM $CaCl_2$ was added. After 1 h, cell extracts were prepared and β-galactosidase activity was measured. Data are averages ± S.D. of three independent experiments.

[17]. Crz1 is phosphorylated and predominantly present in the cytoplasm under normal conditions, whereas, once the cells are treated with $CaCl_2$ the Crz1 is dephosphorylated by CN and subsequently concentrated in the nucleus [18,19, see also Fig. 2A]. The immunosuppressant FK506 is a specific inhibitor of CN [20] and thus it represses the activation of Crz1 by $CaCl_2$. We then determined whether CN and/or Crz1 are involved in the Ca^{2+}-induced expression of *GPX2*. As shown in Fig. 3A, the Ca^{2+}-induced expression of *GPX2-lacZ* was repressed by the addition of 1 μg/ml FK506. Essentially, the same results were obtained by Northern blotting analysis (Fig. 3B). In addition, the Ca^{2+}-induced expression was observed in neither the *crz1*Δ mutant nor *cnb1*Δ (CN deficient) mutant (Fig. 2B). These results strongly suggest that the CN/Crz1-mediated pathway regulates the expression of *GPX2* in response to Ca^{2+}.

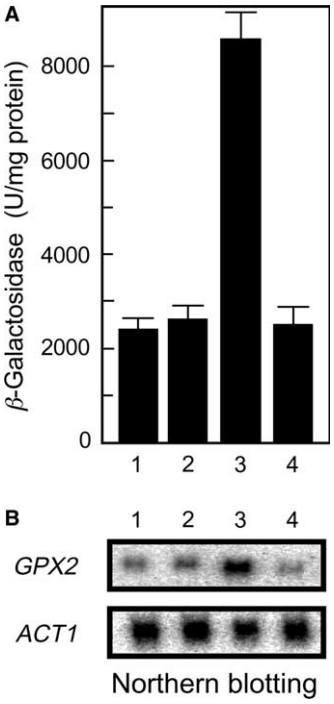

Fig. 3. Ca^{2+}-induced expression of *GPX2* is mediated by the CN/Crz1 pathway. (A) Cells carrying *GPX2-lacZ*-1 were cultured in YPD medium until the exponential phase and treated as follows: slot 1, no chemicals; slot 2, 1 μg/ml FK506; slot 3, 200 mM $CaCl_2$; slot 4, 1 μg/ml FK506 + 200 mM $CaCl_2$. After 1 h, β-galactosidase activity was measured. Data are averages ± S.D. of three independent experiments. (B) Northern blotting was performed using the total RNAs prepared from cells treated with each chemical for 30 min as described above. To each lane, 20 μg RNA was loaded.

3.3. *Crz1-binding region within the GPX2 promoter*

Next, we searched for the Ca^{2+}-responsive *cis*-element within the *GPX2* promoter. As a first approach, we compared the response to Ca^{2+} treatment of a series of promoter-deleted *GPX2-lacZ* reporter genes. Deletion of the 5′-flanking region of *GPX2* from −709 to −485 did not affect the responsiveness to Ca^{2+} (Fig. 4A), although deletion to −306 (*GPX2-lacZ*-3) enhanced the basal expression levels of *GPX2*. The basal expression level of *GPX2* reverted to the wild-type level by further deletion (*GPX2-lacZ*-4). Interestingly, the responsiveness to Ca^{2+} of the *GPX2-lacZ*-3, *GPX2-lacZ*-3a and *GPX2-lacZ*-4 was higher than that of *GPX2-lacZ*-1 possessing the full-length promoter (Fig. 4A), although the Ca^{2+}-induced expression of these reporter genes with deleted promoters was inhibited by FK506. In addition, the disruption of *CRZ1* completely repressed the Ca^{2+}-dependent induction of such reporter genes (data not shown). Further deletion of the *GPX2* promoter abolished the Ca^{2+}-induced expression of *GPX2* (*GPX2-lacZ-5*, Fig. 4A). These results suggest that the *cis*-acting element for Crz1-mediated Ca^{2+} signaling is likely to be located between −204 and −133 within the *GPX2* promoter. In addition, these results somehow imply that the URS(s) (upstream repression sequence), which represses basal expression level as well as responsiveness of Ca^{2+} signaling of *GPX2*, may exist between −485 and −306, although this time we do not focus on such element(s) here.

Recently, Yoshimoto et al. [17] reported a genome-wide analysis of gene expression that is regulated in a CN/Crz1-dependent manner in *S. cerevisiae*. As a result of screening, 163 genes were identified to be regulated by the CN/Crz1-mediated pathway. By computational analysis, the CN/Crz1-dependent genes contain a common sequence (5′-GAGGCTG-3′), which was designated CDRE (CN-dependent response element) [18], in their promoter region. Thus, we looked for this motif between −204 and −133 within the *GPX2* promoter, and found a similar sequence (5′-$^{-151}$CAGGCTG^{-157}- 3′) on the non-coding strand.

To explore whether this motif functions as a Crz1-binding site, EMSA was performed with a DNA probe corresponding to the region from −170 to −147 where the CDRE-like sequence is involved. As shown in Fig. 4B, a band shift was observed when the cell extracts of *crz1*Δ cells expressing GFP-Crz1 were used but not in the case of the vector control. This band disappeared when an excess amount of unlabeled probe was added, suggesting that Crz1 can bind to the CDRE-like sequence in the *GPX2* promoter.

3.4. *Distinct regulation of GPX2 by oxidative stress and Ca^{2+} signaling*

The results we obtained in this study strongly imply that the expression of *GPX2* is independently regulated by oxidative stress in a Yap1/Skn7-dependent manner and by Ca^{2+} signaling by the CN/Crz1-mediated pathway. Crz1 is not involved in the H_2O_2-induced expression of *GPX2*, because GFP-Crz1 was not concentrated in the nucleus under conditions of oxidative stress (Fig. 2A), and the oxidative stress response of *GPX2* was observed in the *crz1*Δ mutant (Fig. 2B). To determine the distinct regulation of *GPX2* by these different signaling pathways, the responsiveness of *GPX2-lacZ* with the full-length promoter was monitored by simultaneous treatment with H_2O_2 and $CaCl_2$. As shown in Fig. 5, an additive effect in terms of the induction of *GPX2* was observed if cells were co-treated with these two chemicals. This reflects the distinct actions of these transcription factors on the *GPX2* promoter in the independent response to oxidative stress and Ca^{2+} signaling (Fig. 6).

4. Discussion

We have demonstrated that the expression of *GPX2* is induced by 200–300 mM $CaCl_2$. This induction was assumed not to be the osmotic stress response, because high concentrations of NaCl (500 mM), KCl (500 mM) and sorbitol (1 M) did not induce the expression of *GPX2* (Fig. 1A). In addition, Ca^{2+}-dependent induction was still observed in mutants defective in *HOG1* and *PBS2*, the gene products of which are required for the osmotic stress response in yeast (data not shown).

Williams and Cyert [21] reported that Skn7 stabilizes Crz1, and therefore, Ca^{2+}-dependent expression of *PMC1* and *FKS2*, both of which are the targets of Crz1, was insufficient in the *skn7*Δ mutant. In contrast to these genes, the expression of *GPX2* following Ca^{2+} treatment was fully induced in the *skn7*Δ (data not shown) and *yap1*Δ*skn7*Δ mutants (Fig. 2B). These observations imply that another transcription factor besides Crz1 might be involved in the expression of *GPX2* in response to Ca^{2+}. For example, it has been reported that Swi5 is able to induce the expression of the CDRE-lacZ reporter gene in *crz1*Δ cells if Swi5 is supplied on a multicopy plasmid [21]. Nevertheless, since the Ca^{2+}-dependent induction of *GPX2* was repressed in cells defective in *CNB1* or *CRZ1*, and in those treated with FK506 (Figs. 2 and 3), we concluded that the CN/

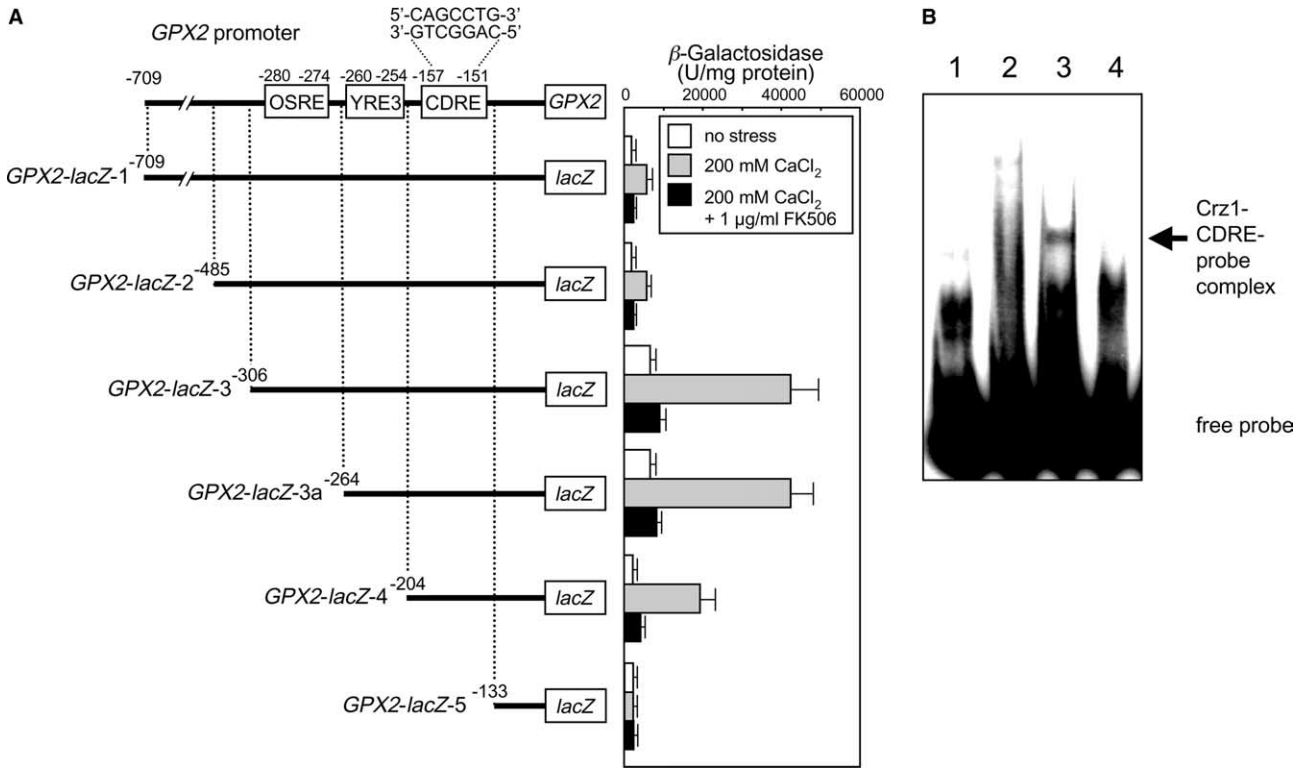

Fig. 4. Identification of a Ca^{2+}-responsive *cis*-acting element within the *GPX2* promoter. (A) The 5′-untranslated region of *GPX2* was deleted as indicated in the figure, then fused with the *lacZ* reporter gene. Cells carrying each reporter gene were cultured until the exponential phase, treated with 200 mM $CaCl_2$ with or without 1 μg/ml FK506 for 1 h, and β-galactosidase activity was measured. Data are averages ± S.D. of three independent experiments. The CDRE (CN-dependent response element)-like sequence was found on the non-coding strand at the position between −157 and −151. OSRE and YRE3 represent the oxidative stress-responsive Skn7 response element and Yap1 response element, respectively [4, also see Fig. 6]. (B) EMSA was carried out using a probe corresponding to the region between −170 and −147 in the *GPX2* promoter, including the CDRE-like motif. Cell extracts used were as follows: lane 1, without cell extract; lane 2, cell extract of the *crz1*Δ mutant carrying empty vector; lane 3, cell extract of the *crz1*Δ mutant carrying pAMS463 (GFP-Crz1); and lane 4, the same cell extracts as in lane 3 with an excess amount (100-fold) of unlabeled probe. Arrow indicates the Crz1-CDRE–probe complex.

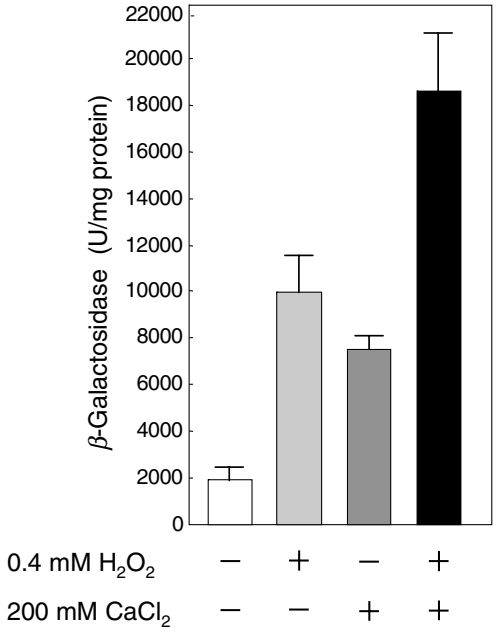

Fig. 5. Additive effect on induction of *GPX2* by H_2O_2 and Ca^{2+}. Cells carrying *GPX2-lacZ*-1 were cultured until the exponential phase, and 0.4 mM H_2O_2 and/or 200 mM $CaCl_2$ were added. After 1 h, cell extracts were prepared and β-galactosidase activity was measured. Data are averages ± S.D. of three independent experiments.

Crz1-mediated Ca^{2+} signaling pathway is the major mechanism by which *GPX2* expression is regulated by Ca^{2+} treatment.

Genome-wide screening using DNA microarray techniques for the yeast genes responsive to Ca^{2+} through the CN/Crz1-mediated pathway revealed that more than 160 genes are regulated by this signaling cascade [17]. Such target genes are classified into several groups, i.e., genes involved in cell wall synthesis, ion/small molecule transport, vesicle transport, lipid and sterol synthesis, degradative enzymes and Ca^{2+} signaling and transcription [22]. Interestingly, *GPX2* as well as other antioxidant genes were not detected in this screening. One possible explanation may be that the changes are below the threshold levels defined [17]. Nevertheless, the Ca^{2+}-dependent induction of *GPX2* is of physiological interest. Gpx2 is a yeast orthologue of mammalian phospholipid hydroperoxide glutathione peroxidase (PHGPx) [23]. Recently, Imai et al. [24] reported that overexpression of PHGPx in mitochondria prevents the opening of mitochondrial permeability transition pores, and the release of cytochrome *c* and apoptosis-inducing factor from mitochondria to block the apoptosis of rat basophil leukemia RBL2H3 cells. In mammalian cells, overload of Ca^{2+} activates CN to dephosphorylate BAD that promotes heterodimerization of BAD with Bcl-xL, thereby allowing translocation to the mitochondria to trigger the apoptotic process [25]. On the other hand, it has been reported that the cell viability of spheroplasts of wild-type *S. cerevisiae* did not

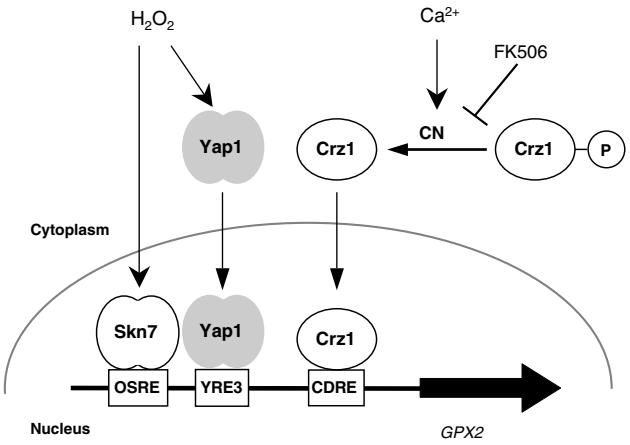

Fig. 6. Summary of the distinct regulation of *GPX2* by oxidative stress and Ca^{2+} signaling. Oxidative stress caused by H_2O_2 results in the nuclear concentration of Yap1 to be bound to the YRE3 (Yap1 response element, 5′-TTAGTAA-3′). The *GPX2* promoter contains three YRE-like elements. Of these, the intrinsic YRE that functions under oxidative stress was identified to be YRE3 [4]. On the other hand, Skn7 always distributes in the nucleus and is bound to the OSRE (oxidative stress-responsive Skn7 response element, 5′-GGCCGGC-3′) [4]. Both Yap1 and Skn7 are required for the oxidative stress response of *GPX2*, although whether there is a direct physical interaction between Yap1 and Skn7 is still under debate. Treatment of yeast cells with $CaCl_2$ activates CN in a Ca^{2+}/calmodulin-dependent manner and the activated CN dephosphorylates Crz1. The dephosphorylated Crz1 is concentrated in the nucleus and is then bound to the CDRE-like sequence (5′-CAGGCTG-3′), thereby activating *GPX2* expression. FK506 is a specific inhibitor of CN, and therefore, FK506 represses the Ca^{2+}-dependent induction of *GPX2*.

drop by treatment with Ca^{2+} alone, although those lacking *TSA1* coding for cytosolic thioredoxin peroxidase (peroxiredoxin) and/or those treated with 3-amino-1,2,4-triazole (catalase inhibitor) were sensitive to Ca^{2+} [26,27]. These results imply that antioxidant enzymes seem to protect yeast cell from the Ca^{2+}-induced growth inhibition. Here, we demonstrated that the yeast PHGPx homologue *GPX2* is induced by both oxidative stress and Ca^{2+} (Fig. 6). To our knowledge, this is the first report proving that the expression of the GPx gene is regulated in a Ca^{2+}/CN-dependent manner in eukaryotes. Elucidation of the physiological significance of the induction of *GPX2* by Ca^{2+} as well as H_2O_2 would expand our understanding of the correlation between oxidative stress response and Ca^{2+} signaling.

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