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Regulation of the yeast phospholipid hydroperoxide glutathione peroxidase *GPX2* by oxidative stress is mediated by Yap1 and Skn7

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Abstract The *GPX2* gene encodes a homologue of phospholipid hydroperoxide glutathione peroxidase in *Saccharomyces cerevisiae*. The *GPX2* promoter contains three elements the sequence of which is completely consistent with the optimal sequence for the Yap1 response element (YRE). Here, we identify the intrinsic YRE that functions in the oxidative stress response of *GPX2*. In addition, we discovered a *cis*-acting element (5′-GGCCGGC-3′) within the *GPX2* promoter proximal to the functional YRE that is necessary for H_2O_2-induced expression of *GPX2*. We present evidence showing that Skn7 is necessary for the oxidative stress response of *GPX2* and is able to bind to this sequence. We determine the optimal sequence for Skn7 to regulate *GPX2* under conditions of oxidative stress to be 5′-GGC(C/T)GGC-3′, and we designate this sequence the oxidative stress-responsive Skn7 response element.

Keywords: Glutathione peroxidase; Yap1; Skn7; Yeast; Oxidative stress

1. Introduction

Accumulation of reactive oxygen species in the cell induces toxic damage to cellular components, such as lipids, DNA and proteins, and it eventually leads to cell death. Therefore, organisms have evolved a number of antioxidant systems to protect themselves from oxidative stress. Glutathione peroxidase (GPX) is one of the important antioxidant enzymes. We have found that *Saccharomyces cerevisiae* has three GPX homologues (encoded by the *GPX1*, *GPX2*, and *GPX3* genes) [1]. Of these *GPX* genes, the expression of *GPX2* was specifically induced by oxidative stress in the presence of Yap1 [1]. Yap1 binds to the specific DNA sequence termed YRE (Yap1 response element, 5′-TTA(C/G)TAA-3′) [2]. In addition to Yapl, Msn2 and Msn4, which are C_2H_2-type zinc-finger transcription factors, are known to be responsible for the stress-induced expression of several genes. Both Msn2 and Msn4 bind to the stress response element (STRE, 5′-AGGGG-3′ or 5′-CCCCT-3′) that can be found within the promoter region of many stress-responsive genes [3]. Genetic interaction between Yapl and Msn2/Msn4 has been reported with respect to the osmotic stress response of *TPS2*. Expression of *TPS2* is induced by several stress stimuli such as heat shock, osmotic stress and metabolic inhibitors [4]. Gounalaki and Thireos [4] reported that Yap1 is required for the transcriptional regulation of *TPS2* by osmotic stress through the STRE in the *TPS2* promoter, which, however, does not contain the YRE. Similarly, Winderickx et al. [5] reported that the oxidative stress-induced expression of *CTT1* requires Yap1 even though the *CTT1* promoter also has no YRE but contains STRE. Therefore, the distinct roles of these transcription factors (Yap1 and Msn2/Msn4) remain to be elucidated.

In addition to Yap1 and Msn2/Msn4, it has been reported that Skn7 also plays a crucial role in the expression of the oxidative stress-responsive genes. Lee et al. [6] performed two-dimensional gel electrophoretic analysis to screen yeast proteins that can be induced by H_2O_2, and classified Yap1-, Skn7- and Yap1/Skn7-dependent genes. Out of 32 Yap1-dependent genes that they identified, approximately one-third required both Yap1 and Skn7. In addition, half of these Yap1-dependent genes do not possess YRE within their promoter region. On the other hand, the consensus sequence of the *cis*-acting element for Skn7 under oxidative stress-induced conditions has not yet been identified.

In this study, we focused on the transcriptional regulation of *GPX2* that contains both YRE and STRE in its promoter region. We reveal that both Yap1 and Skn7 are essential for the oxidative stress response of *GPX2,* but Msn2 and Msn4 are not. We also discovered a *cis*-acting element for Skn7 for oxidative stress response proximal to the functional YRE within the *GPX2* promoter.

2. Materials and methods

2.1. Strains

All yeast strains of *S. cerevisiae* used in this study have the YPH250 background (*MAT***a** *trp1-Δ1 his3-Δ200 leu2-Δ1 lys2-801 ade2-101 ura3-52*). The *msn4Δ::ADE2* allele in SCM241 [7] was amplified by PCR to disrupt the *MSN4* locus of YPH250. Disruption of *SKN7* was carried out using the *skn7Δ::TRP1* plasmid [8]. The *yap1Δ::HIS3* and *msn2Δ::HIS3* mutants in the YPH250 background were described previously [9]. YPH252 is an isogenic strain of YHP250 with a different

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mating type (*MAT*α). YPH499 (*MAT***a** *trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 lys2-801 ade2-101 ura3-52*), its isogenic strain with a different mating type, YPH500 (*MAT*α) and W303-1a (*MAT***a** *trp1-1 his3-11, 15 leu2-3, 112 ade2-1 ura3-1 can1-100*) were used as necessary.

2.2. Western blotting

The *GPX2* gene was expressed in *Escherichia coli* and Gpx2 was purified to immunize New Zealand White rabbits to raise anti-Gpx2 antibody. Yeast cellular proteins were separated by SDS–PAGE and the separated proteins were electrically transferred to PVDF membrane (Immobilon; Millipore). Anti-Gpx2 antiserum was used as the primary antibody and anti-rabbit IgG antibody conjugated with horseradish peroxidase (New England Biolabs) was used as the secondary antibody. Immunoreactive protein was visualized by 4-chloro-1-naphtol and H_2O_2.

2.3. Construction of the GPX2–lacZ reporter gene

A *GPX2–lacZ* fusion plasmid was constructed using a PCR-amplified DNA fragment (−709/+7) containing the 5′-flanking region of *GPX2* and the sequence encoding the first 2 amino acids of the Gpx2 protein. The forward (GPX2-1, 5′-TTACCGTTGTCGACCTTGCTCTAC-3′) and reverse (GPX21acR2, 5′-TCATAAAGAATTCTGGTCATTTTGAATTAT-3′) primers were designed to contain *Sal*I (forward) and *Eco*RI (reverse) sites, respectively. 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.

To construct the *gpx2-lacZ*ΔGCRE, the 5′ upstream region of the GC-rich element (GCRE, from −709 to −281) and the 3′ downstream region (from −272 to +7) of the GCRE in *GPX2* were amplified by PCR using the following combination of oligonucleotides: GPX2-1 plus npalR (5′-CTAGATATGGATCGATGAGTAGCCT-3′), and GPX21acR2 plus npalF (5′-GCTACTCGGATCGATATATCTAGACA-3′). Each PCR product was mixed, digested with *Cla*I, *Sal*I and *Eco*RI followed by ligation, and then the generated fragment was cloned into the *Sal*I–*Eco*RI site of YIp358R.

To construct the GCRE-$CYC1_{TATA}$-*lacZ* reporter gene, complementary oligonucleotides (GP2PAL-1, 5′-ACTCGAGACTCGGCCGGCCATATCTCGAGT-3′ and GP2PAL-2, 5′-TGAGCTCTGAGCCGGCCGGTATAGAGCTCA-3′) that correspond to the region from −284 to 269 within the *GPX2* promoter were annealed, digested with *Xho*I (the *Xho*I site is underlined) and the resultant fragment was cloned into the *Xho*I site of pTBA30 which contains $CYC1_{TATA}$-*lacZ* without its original upstream activation site (UAS) [10].

2.4. Mutagenesis of the GPX2 promoter

PCR-based site-directed mutagenesis was employed to introduce point mutations into the YRE and GCRE within the *GPX2* promoter. The first PCR was performed with the following primer combinations: GPX2-1 plus the mutagenesis primer (reverse primer; e.g., mYRE1R) and GPX21acR2 plus the mutagenesis primer (forward primer; e.g., mYRE1F). Mutagenesis primers are listed in Table 1. The last letter of each primer in Table 1 represents the direction of the primer; i.e., F and R donate forward and reverse, respectively. The plasmid carrying the region from −709 to +7 of the *GPX2* gene in YIp358R was used as template DNA. The PCR products amplified by each primer set were combined, subjected to the second PCR with GPX2-1 and GPX21acR2, digested with *Sal*I and *Eco*RI, and then introduced into the *Sal*I–*Eco*RI site of YIp358R to generate pmYRE1, pmYRE2, pmYRE3 and a series of pmGCREs. A plasmid carrying two or three mutations in each YRE was constructed using the same procedure, except that pmYRE1 or pmYRE23 was used as the template.

2.5. β-Galactosidase assay

Cells were cultured in YPD medium until log phase and treated with H_2O_2. The preparation of cell extracts and assay of β-galactosidase activity were carried out as described previously [9]. 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 [11].

Table 1
PCR primers used for construction of a series of *GPX2–lacZ* reporter plasmids

Primer	Sequence
mYRE1F	5′-CGCGCTGTTAAGGTAAGCATTTTTCGAGAT-3′
mYRE1R	5′-ATCTCGAAAAATGCTTACCTTAACAGCGCG-3′
mYRE2F	5′-CGGACGTTACTAACGTAATGTACGACGAAC-3′
mYRE2R	5′-GTTCGTCGTACATTACGTTAGTAACGTCCG-3′
mYRE3F	5′-CTAGACAATAAGGTATTCCGATGACAAAGA-3′
mYRE3R	5′-TCTTTGTCATCGGAATACCTTATTGTCTAG-3′
GCREct-F	5′-GGCTACTCGGCTGGCCATATC-3′
GCREct-R	5′-GATATGGCCAGCCGAGTAGCC-3′
mGCtrx-F	5′-GGCTACTATGCCGGCCATATC-3′
mGCtrx-R	5′-GATATGGCCGGCATAGTAGCC-3′
GCREc1a-F	5′-GCTACTCGGCAGGCCATAT-3′
GCREc1a-R	5′-ATATGGCCTGCCGAGTAGC-3′
GCREc1g-F	5′-GCTACTCGGCGGGCCATAT-3′
GCREc1g-R	5′-ATATGGCCCGCCGAGTAGC-3′
GCREc2g-F	5′-GCTACTCGGCCGGGCATAT-3′
GCREc2g-R	5′-ATATGCCCGGCCGAGTAGC-3′
GCREc2a-F	5′-GCTACTCGGCCGGACATAT-3′
GCREc2a-R	5′-ATATGTCCGGCCGAGTAGC-3′
GCREc2t-F	5′-GCTACTCGGCCGGTCATAT-3′
GCREc2t-R	5′-ATATGACCGGCCGAGTAGC-3′
GCRE-R-F	5′-GCTACTCGCCGGCCCATAT-3′
GCRE-R-R	5′-ATATGGGCCGGCGAGTAGC-3′

2.6. In vitro synthesis of Yap1 and Skn7

The coding region of *SKN7-6Myc* was amplified by PCR using the pSKN7-6Myc plasmid (pRS416 + *SKN7-6Myc*) [12] as a template with the following primers: SKN7F, 5′-TTTTTTCGAAGCTTACTTTTGATATCC-3′ and SKN7R, 5′-CTCACTAAAGGGAACAAAAGCTG-3′. The SKN7F primer was designed to contain a *Hin*dIII site (underlined), while the SKN7R primer corresponds to the multicloning site of pRS416. The amplicon was digested with *Hin*dIII and *Sac*I, then cloned into the *Hin*dIII–*Sac*I site of the Luciferase T7 control plasmid (Promega, Madison, WI). The resultant plasmid was designated pT7-SKN7-6Myc.

YAP1 was amplified by PCR with primers YAP1FC (5′-GAAGATCTATGAGTGTGTCTACCGCCAA-3′) and YAP1RC (5′-TCCATAAAGTTGCGGCCGCCGTTCATATGC-3′). *Bgl*II and *Not*I sites were designed in the YAP1FC and YAP1RC, respectively (underlined). The amplified fragment was digested with *Bgl*II and *Not*I, and cloned into the *Bgl*II–*Not*I site of the 3xHA-tagging plasmid pSLF172 (ATCC 87609). The resultant plasmid was digested with *Bgl*II and *Sma*I, and the *YAP1-3HA* fragment was cloned into the *Bam*HI–*Sac*I site of the Luciferase T7 control plasmid (Promega). The *Sac*I site of the vector was filled with Klenow before ligation of the *YAP1-3HA* fragment. The plasmid obtained was named pT7-Yap1-3HA.

Both Skn7-6Myc and Yap1-3HA were synthesized in vitro using the T7 RNA polymerase and rabbit reticulocyte lysate kit (Promega). Procedures for in vitro transcription and translation were followed according to the manufacturer's specification. Translated products were verified by Western blotting using anti-c-Myc polyclonal antibody (A-14, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-HA monoclonal antibody (12CA5, Roche Diagnostics, Basel, Switzerland).

2.7. Electrophoretic mobility shift analysis

Oligonucleotide probes containing the various target sites were generated by PCR amplification. To amplify the GCRE + YRE3 region (−297/−229), GPX2-S (5′-CCATCGATTTTTTTAGAGGCTACTCGGCC-3′) and GPX2-AS (5′-GACTAGTGGTTTTAAACTCTTTGTCATCGG-3′) were used. A *Cla*I site and *Spe*I site (underlined) were designed in GPX2-S and GPX2-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-GCRE + YRE3) was digested with *Cla*I and *Spe*I, and the 3′-end of the 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. To prepare the ^{32}P-labeled probes for GCRE and

YRE3, the pRS416-GCRE + YRE3 plasmid was digested with *Cla*I and *Xba*I (GCRE probe) or with *Xba*I and *Spe*I (YRE3 probe), and the 3′-end of each DNA fragment was labeled as described above. The *Xba*I site (−269/−264) is present between GCRE (−279/−273) and YRE3 (−260/−254).

The DNA binding reactions were carried out in the binding buffer [25 mM Tris–HCl (pH 7.5), 50 mM NaCl, 2 mM EDTA, 7 mM $MgCl_2$, and 10% glycerol] with 20 μg of protein from the cell extract of yeast or 1 μl of in vitro-synthesized products, 2 ng of ^{32}P 3′-end-labeled probes and 1 μg of poly(dI–dC) in a total volume of 20 μl for 15 min at room temperature and then for another 15 min on ice. The resultant mixture was subjected to non-denaturing polyacrylamide gel (4%) electrophoresis in 0.5 × TBE for 1 h at 200 V. The gel was dried onto Whatman 3MM paper and an autoradiograph was taken using a Fujix Bio-Imaging Analyzer BAS2000 (Fuji Photo Film, Tokyo, Japan).

3. Results

3.1. Msn2 and Msn4 are not essential for the oxidative stress response of GPX2

The *GPX2* promoter contains several potential *cis*-elements that may be involved in the oxidative stress response in yeast; i.e., three YREs (YRE1, $^{-627}$TTAGTAA^{-621}; YRE2, $^{-417}$TTACTAA^{-411}; and YRE3, $^{-260}$TTAGTAA^{-254}) and STRE ($^{-166}$CCCCT^{-162}). Previously, we have reported that Yap1 is required for the oxidative stress response of *GPX2* [1]. Regarding the activation of Yap1 under conditions of oxidative stress, Gpx3 and Ybpl were found to play crucial roles [13,14]. We cloned the *GPX3* gene which encodes a homologue of GPX in yeast [1], and recently, Delaunay et al. reported that Gpx3 functions as a redox transducer for Yap1 [13]. Indeed, H_2O_2-induced expression of *GPX2* did not occur in a *gpx3*Δ mutant (data not shown). On the other hand, Veal et al. reported that Ybpl functions in the activation of Yap1 in the Gpx3 pathway [14]. They reported that Ybpl binds to Yap1 in vivo. In addition, these authors also reported that a yeast strain W303-1a carries *ybp1-1* mutation that abolishes the function of Ybp1 [14]. We compared the responsiveness of *GPX2* to oxidative stress in some typical laboratory yeast strains, and found that the induction was insufficient in the W303-1a strain (Fig. 1). These results indicate that the expression of *GPX2* is dependent upon the Gpx3–Ybp1–Yap1 pathway.

In addition to Yap1, here we determined whether Msn2 and Msn4 are also involved in the oxidative stress-induced expression of this gene by monitoring the expression of *GPX2* in *msn*2Δ, *msn*4Δ and *msn*2Δ*msn*4Δ mutants. The expression of *GPX2* by 0.4 mM H_2O_2 treatment was still induced in a *msn2*Δ*msn4*Δ mutant (data not shown). Therefore, these two transcription factors seem not to be involved in the oxidative stress response of *GPX2*, and the STRE found within the promoter region of this gene may not function as a *cis*-element at least under conditions of oxidative stress.

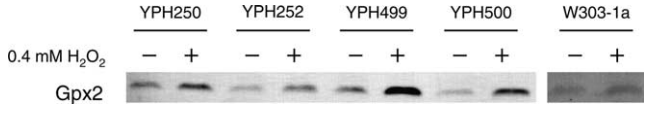

Fig. 1. Induction of Gpx2 production in different laboratory strains. Cells were cultured until log phase and treated with 0.4 mM H_2O_2 for 1 h. Gpx2 protein levels in each strain were detected by Western blotting with an anti-Gpx2 antibody. To detect Gpx2 in W303-1a, we applied higher amount of proteins of W303-1a to the SDS–PAGE than proteins from other yeast strains, and the membrane was stained for longer period to visualize Gpx2 band in the staining mixture, because the basal levels of Gpx2 in W303-1a were lower than those in other strains. Subsequently, background for W303-1a was increased.

3.2. Identification of the oxidative stress-responsive YRE in the GPX2 promoter

To identify the functional YRE, a point mutation was introduced into each potential YRE (5′-TTA(G/C)TAA-3′ → 5′-T<u>A</u>A(G/C)<u>GT</u>A-3′) [15,16]. We constructed a *GPX2–lacZ* reporter gene that contains the region from −709 to +7 of *GPX2* to quantify the induction of *GPX2*. As shown in Fig. 2, introduction of a mutation into the YRE1 (m1) and YRE2 (m2) did not affect the induction of *GPX2* expression by oxidative stress, whereas the mutation of YRE3 (m3) reduced the basal expression levels of *GPX2–lacZ* and completely diminished the oxidative stress-dependent induction. The *GPX2–lacZ* reporter gene carrying the mutated YRE1 and YRE2 (m12) could still respond to H_2O_2, whereas no induction was observed if the mutated YRE3 was present (m13, m23 and m123). These results clearly indicate that only YRE3 functions as the oxidative stress-responsive *cis*-element within the *GPX2* promoter. One of the possible explanations for the difference in ability of each YRE as *cis*-element may be the distance from the TATA box. Therefore, although these reporter genes were inserted into the *URA3* locus, essentially the same tendency would be obtained if the mutation of YRE3 was inserted into the *GPX2* locus.

3.3. A new cis-acting element necessary for the oxidative stress response of GPX2

Next, we searched the characteristic DNA sequence proximal to the YRE3 to find the factor(s) that may be involved in the oxidative stress response of *GPX2* together with Yap1, and a short palindromic DNA sequence enriched in G and C was found 13-nucleotides upstream of the YRE3. We temporarily designated this sequence the GCRE (from −280 to −273; 5′-GGCCGGCC-3′). To determine whether the GCRE corresponds to oxidative stress, this element was deleted from the *GPX2* promoter (*gpx2-lacZ*ΔGCRE). As shown in Fig. 3A, the strain carrying *gpx2-lacZ*ΔGCRE was not able to respond to H_2O_2. To confirm whether the GCRE itself can function as an oxidative stress-responsive *cis*-acting element, this short sequence was introduced upstream of the *CYC1*$_{TATA}$-*lacZ* reporter gene (Fig. 3B). Although this UAS-less reporter gene slightly responds to H_2O_2, expression of the GCRE-*CYC1*$_{TATA}$-*lacZ* reporter gene was markedly induced by H_2O_2. Additionally, we found that the oxidative stress-induced expression of this reporter gene, as well as that of *GPX2–lacZ*, was not seen in the *skn7*Δ mutant, suggesting that Skn7 is involved in the oxidative stress response of *GPX2* via GCRE. Intriguingly, no induction of GCRE-*CYC1*$_{TATA}$-*lacZ* expression was seen in the *yap1*Δ mutant even though the nucleotide sequence of the GCRE does not resemble that of YRE.

3.4. GCRE functions as a cis-element for Skn7 under conditions of oxidative stress

Morgan et al. reported that Skn7 binds to the promoter region of *TRX2* at the position between −164 and −142 [17]. To specify the Skn7-binding sequence, we searched for the GCRE-like sequence and found that a similar sequence (5′-GGCtGGC-3′; lower case letters indicate from the nucleotides which are different in the GCRE in the *GPX2* promoter) is present on the non-coding strand within this region. These authors also reported that Skn7 cannot bind to the

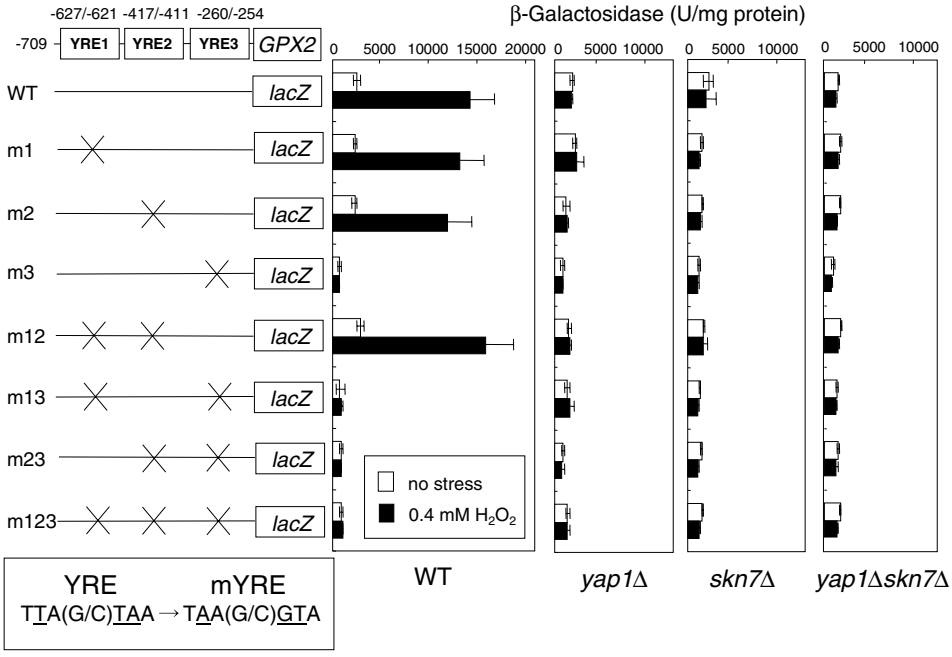

Fig. 2. Identification of a functional YRE within the *GPX2* promoter. Cells carrying each *GPX2–lacZ* reporter gene were cultured until log phase and treated with 0.4 mM H_2O_2 for 1 h.

5′-aTGCtGGC-3′ sequence (mutations are underlined) [17]. To determine whether this is also the case for the GCRE in the *GPX2* promoter, point mutations were introduced into the GCRE as indicated in Fig. 4. As a result, alteration of GCRE to the *TRX2-type* sequence (5′-GGCCGGC-3′ → 5′-GGCTGGC-3′, mGCRE-1) did not affect the responsiveness to oxidative stress, whereas the mutation that diminishes the function of the *TRX2* promoter (mGCRE-2, 5′-aTGCCGGC-3′) also impaired the ability of the *GPX2* promoter to respond to H_2O_2.

We then tried to determine the optimal sequence for Skn7 to respond to H_2O_2. We screened several antioxidant genes that require both Yap1 and Skn7 for induction under conditions of oxidative stress for the presence of a GCRE-like sequence within the promoter region of such genes, and found a similar sequence in the promoter region of *TSA1* ($^{-129}$GGCTGGC^{-123}), *CCP1* ($^{-245}$GGCCGGC^{-239}) and *TRR1* ($^{-210}$GGCTGGG^{-204}), in addition to *TRX2* ($^{-158}$GGCTGGC^{-152}). By comparing these GCRE-like sequences, a conserved motif (5′-**GGC**(C/T)**GG**(C/G)-3′) is present, with the first three bases as well as the 5th and 6th bases being highly conserved (bold face letters). Thus, we changed the nucleotide at the 4th and 7th positions as indicated in Fig. 4. Alteration of the nucleotide at the 4th position (C) to "A" (mGCRE-3, 5′-GGCAGGC-3′) or "G" (mGCRE-4, 5′-GGCGGGC-3′) abolished induction of *GPX2* expression in response to H_2O_2, whereas changing it to "T" (mGCRE-1, *TRX2-type*) did not affect the oxidative stress response as described above. In the case of the 7th position, the C→T mutation (mGCRE-7, 5′-GGCCGGT-3′) completely repressed the induction of *GPX2* expression, while the C→G (mGCRE-5, 5′-GGCCGGG-3′) or C→A (mGCRE-6, 5′-GGCCGGA-3′) mutations strongly repressed the induction but such mGCREs can still partially respond to oxidative stress (Fig. 4). Taken together, we concluded that the optimal sequence for the GCRE is 5′-GGC(C/T)GGC-3′. However, when we put this optimal GCRE on the non-coding strand of the *GPX2* promoter (mGCRE-8, 5′-GCCGGCC-3′), it did not function as a *cis*-element for the oxidative stress response of *GPX2,* in contrast with the case of *TRX2*.

3.5. DNA binding property of Yap1 and Skn7

We have demonstrated that both YRE3 and GCRE function as *cis*-elements for the oxidative stress response of *GPX2*. Thus, we then needed to determine whether Yap1 and Skn7 can bind to the YRE3 and GCRE, respectively. To address this issue, we performed a gel-shift assay using Yap1 and Skn7 produced by the rabbit reticulocyte in vitro transcription–translation system. As shown in Figs. 5A and B, both Yap1 and Skn7 could bind to the GCRE + YRE3 probe (−297/−229). The bands corresponding to the Skn7-probe (GCRE + YRE3) complex disappeared when an excess amount of unlabeled GCRE (wild-type) was added (Fig. 5A). Similarly, this bandshift was apparently suppressed if cold mGCRE-1 probe (*TRX2-type*) was added as a competitor. However, no competition occurred when mGCRE-2, which cannot respond to oxidative stress (see Fig. 4), was used. These results suggest that Skn7 can bind to both the wild-type GCRE as well as the *TRX2-type* mGCRE-1. This was also the case for the binding profile of Yap1 to the GCRE+YRE3 probe (Fig. 5B). The bandshift of the Yap1-probe complex was repressed by the wild-type YRE3 (5′-TTAGTAA-3′) but not by the mutant YRE3 (5′-TAAGGTA-3′, mYRE3). These results suggest the specific binding of Skn7 to GCRE and Yap1 to YRE3. To confirm this, an electrophoretic mobility shift assay (EMSA) was carried out using probes corresponding to GCRE (−297/−269) and YRE3 (−268/−229) with Skn7 or Yap1. As shown in Fig. 5C, Skn7 bound to the GCRE probe but not to the YRE3 probe, while Yap1 bound to the YRE3 probe but not to the GCRE probe. These band shifts disappeared following the addition of each non-labeled probe (data not

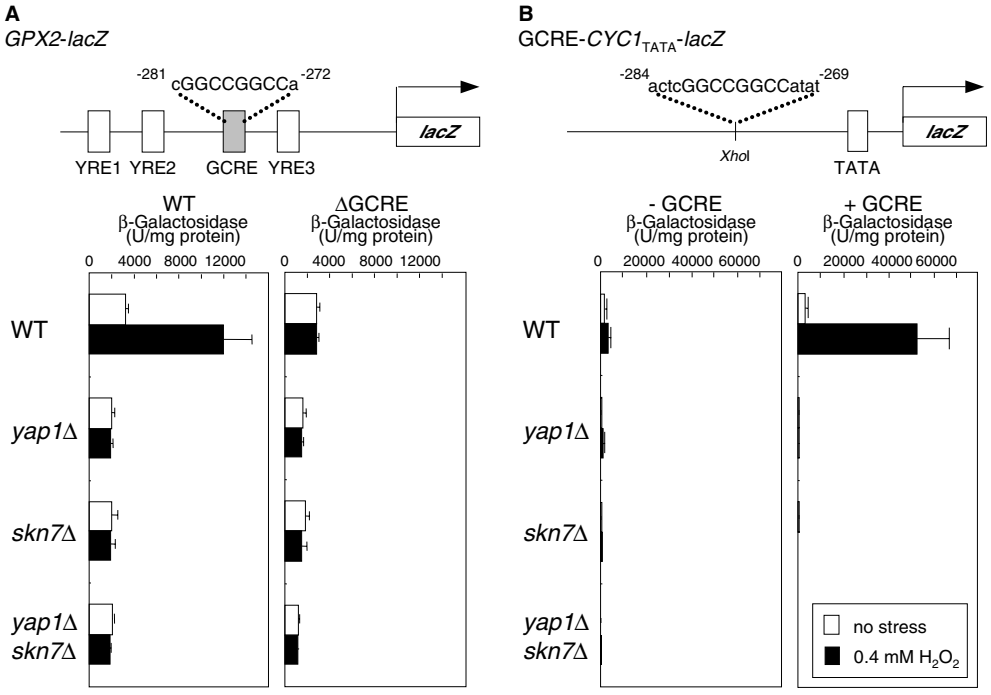

Fig. 3. Effects of GCRE on the response to H_2O_2. (A) Nucleotides at the position between −280 and −273 were deleted from *GPX2–lacZ* to construct *gpx2-lacZ*ΔGCRE. (B) Oligonucleotides corresponding to the *GPX2* promoter from −284 to −269, including the GCRE, were inserted into the *Xho*I site located upstream of the UAS-less *CYC1*$_{TATA}$-*lacZ* reporter gene.

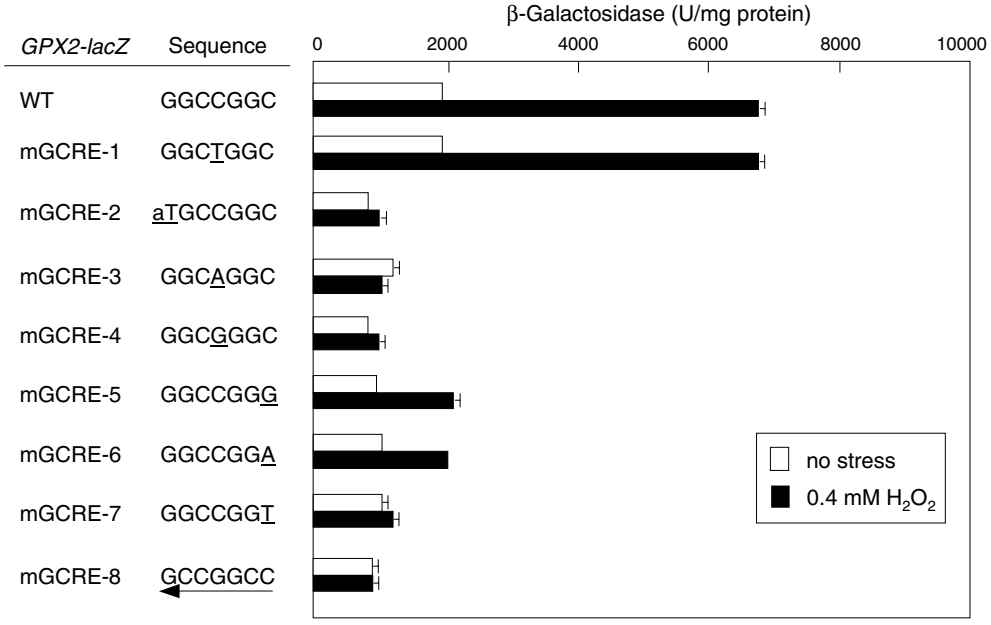

Fig. 4. Functional analysis of GCRE. A point mutation was introduced into the GCRE in the *GPX2–lacZ* reporter gene as indicated in the figure. Underlining indicates the altered nucleotide. In the case of mGCRE-8, the wild-type GCRE (5′-GGCCGGC-3′) was put on the non-coding strand at the same position as on the coding strand. The arrow indicates the direction of the wild-type GCRE on the non-coding strand. Cells carrying each mutant *GPX2–lacZ* reporter gene were treated with or without 0.4 mM H_2O_2 for 1 h and the *β*-galactosidase activity was measured.

shown). Taken together, we concluded that Yap1 and Skn7 bind to the YRE3 and GCRE, respectively.

3.6. Responsiveness of Skn7-binding site

Skn7 possesses a characteristic domain, referred to a receiver domain, that is found in proteins constituting a two-component system (Asp–His phosphorelay protein) [17,18]. Under hypotonic conditions, Asp427 of Skn7 receives phosphorus group from Ypd1, a phosphorelay protein, and phospho-Skn7 activates hypo-osmotic stress responsive genes such as *OCH1* [19]. In the case of oxidative stress response of Skn7, phosphorylation of Asp427 is dispensable [15,20]. We

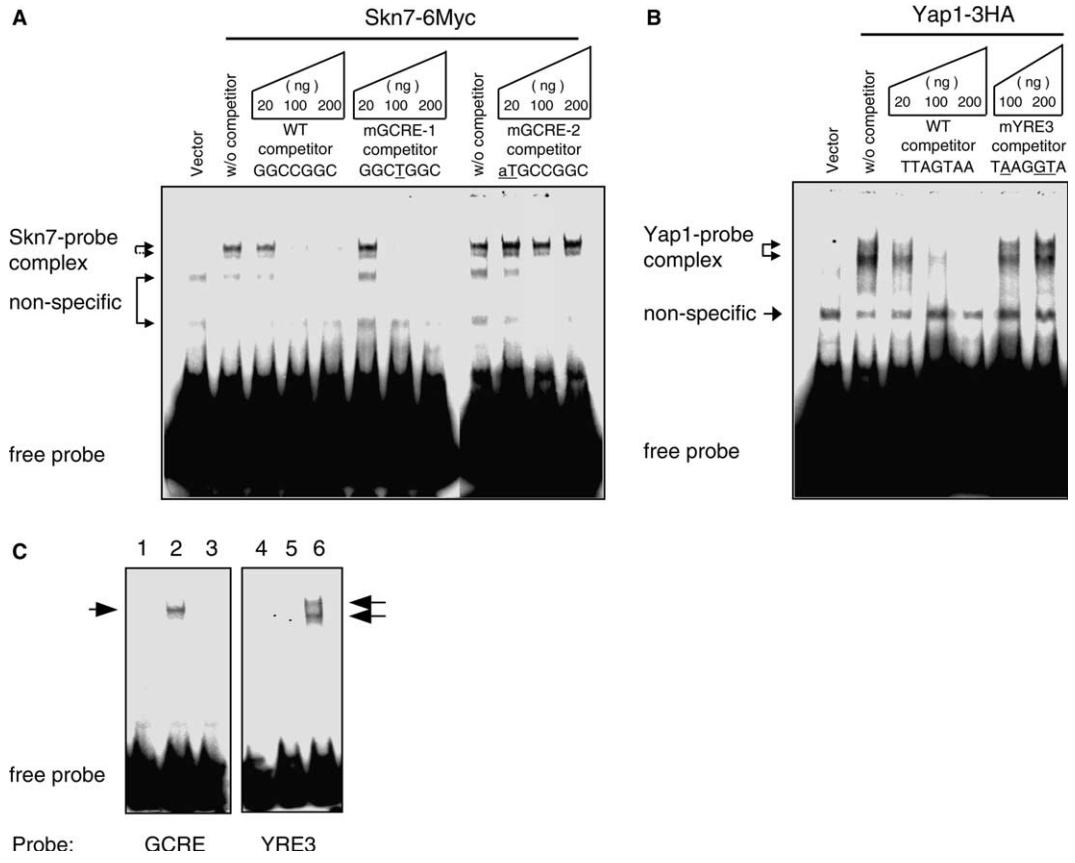

Fig. 5. Electrophoretic mobility shift assay. Detailed conditions for the EMSA were described in Section 2. Probes used correspond to the regions as follows: between −297 and −229 (GCRE+YRE3 probe) (A and B); between −297 and −269 (GCRE probe) (C, left panel); and between −268 and −229 (C, right panel). For control experiments for the EMSA (lane, vector in A and B), the vector alone was used in place of pT7-SKN7-6Myc or pT7-Yap1-3HA in the in vitro transcription and translation mixture, and the resultant reaction mixture was subjected to EMSA. (A) In vitro-synthesized Skn7-6Myc was used. The shifted band disappeared following the addition of excess (20 ng, 10-fold; 100 ng, 50-fold; and 200 ng, 100-fold) unlabeled wild-type GCRE (5′-GGCCGGC-3′) or mGCRE-1 (5′-GGCTGGC-3′), which can both respond to H_2O_2, but not by mGCRE-2 (5′-aTGCCGGC-3′) that cannot induce *GPX2* expression (see Fig. 4). (B) In vitro-synthesized Yap1-3HA was used. The band corresponding to the Yap1-probe complex disappeared following the addition of wild-type YRE3 (5′-TTAGTAA-3′) but not by that of mYRE3 (5′-TAAGGTA-3′) that abolishes the oxidative stress response of *GPX2* (see Fig. 2). (C) The GCRE probe and YRE3 probe were used for the EMSA in the left panel and right panel, respectively. Lanes 1 and 4, no proteins were added; lanes 2 and 5, Skn7-6Myc was added; and lanes 3 and 6, Yap1-3HA was added to the EMSA mixture. Arrows indicate the protein-probe complex.

confirmed that the oxidative stress-induced expression of *GPX2* occurred when an $Skn7^{D427N}$ mutant was expressed in the *skn7*Δ mutant (data not shown). Li et al. reported that *OCH1* promoter contains the SSRE [*sln1* star (*sln1**) response element: 5′-ATTTGGC(C/T)GG(C/G)C-3′] to which Skn7 can bind [18]. The SSRE sequence includes the optimal GCRE [5′-GGC(C/T)GGC-3′] that we identified within the *GPX2* promoter in this study. However, as far as we examined, *GPX2* was not induced by hypo-osmotic stress. Similarly, *OCH1* did not respond to H_2O_2 (data not shown). Considering the correlation between Yap1 and Skn7 for oxidative stress response of *GPX2,* the GCRE may not be sufficient for Skn7 to regulate *GPX2* under hypo-osmotic conditions. It is not surprising that the *OCH1* promoter has different *cis*-acting element(s) yet to be discovered for hypotonic stress response. Alternatively, 5′ sequence of SSRE (5′-ATTT-3′) might be necessary for the hypo-osmotic stress response. To distinguish the responsiveness of SSRE (hypotonic stress) and GCRE (oxidative stress), we designated the latter the oxidative stress-responsive Skn7 response element (OSRE).

4. Discussion

Since the disruption of either *YAP1* or *SKN7* enhances the susceptibility to H_2O_2, it is obvious that both transcription factors play a crucial role in the adaptation to oxidative stress. Nevertheless, the relationship between these two factors in the induction of the expression of antioxidant genes remains under debate. We have demonstrated that both Yap1 and Skn7 are essential for the oxidative stress-induced expression of *GPX2* (Fig. 2A), whereas the induction of *TRX2* expression by H_2O_2 still occurred in a single mutant of *YAP1* or *SKN7* [17]. Contrary to the synergistic behavior of Yap1 and Skn7 in relation to *GPX2* and *TRX2*, a confusing fact is that the H_2O_2-induced expression of *GSH1* and *GLR1*, both of which are Yap1 target genes, is accelerated in an *skn7*Δ mutant [6]. This indicates that Skn7 is a negative regulator of Yap1 at least for the expression of *GSH1* and *GLR1*. However, regardless of how Skn7 regulates Yap1; i.e., negatively or cooperatively, a certain physical interaction between Yap1 and Skn7 is expected. Yap1 shuttles between the cytoplasm and nucleus under non-stress conditions, but is concentrated

in the nucleus under conditions of oxidative stress [21]. On the other hand, Skn7 is constitutively present in the nucleus [22]. Thus, a feasible model for the induction of *GPX2* expression is that Skn7, already bound to the OSRE, recruits Yap1 to the YRE3. To investigate this possibility, we added an in vitro-synthesized Yap1 to the EMSA mixture containing Skn7 and the OSRE probe-complex to determine whether a super-shifted band could be seen through a direct physical interaction between Yap1 and Skn7. However, we could not detect a super-shifted band under the conditions we tested (data not shown). Morgan et al. reported that they failed to observe a direct interaction between Yap1 and Skn7 by co-immunoprecipitation analysis [17]. On the other hand, Lee et al. performed an EMSA using the cell extracts of cells overproducing Yap1 and Skn7 and a relatively long probe expanding from position −204 to +1 of *TSA1*. They reported that both Yap1 and Skn7 bound to this region independently [6]. The *TSA1* promoter contains an OSRE-like sequence but no YRE.

Juhnke et al. reported that the *FAP7* gene product is required for the oxidative stress response of *TSA1* [22]. To investigate whether such factors (e.g., Fap7 and Ybp1) are involved in the physical interaction between Yap1 and Skn7 *in vitro,* we added the cell extracts of the *yap1*Δ*skn7*Δ mutant that had and had not been exposed to oxidative stress, to the EMSA mixture where both in vitro-synthesized Yap1 and Skn7 were present. However, a super-shifted band was not detected (data not shown).

We could not obtain evidence for the existence of another factor(s) that may mediate a physical interaction between Yap1 and Skn7 in vitro, but we cannot exclude the possibility of the existence of such a factor(s) or a physical interaction between Yap1 and Skn7. In addition, since Yap1 participates in the regulation of not only antioxidant genes but also several drug resistance genes [23–25], and this transcription factor regulates the genes that do not have YRE in their promoter regions [4–6], it may be feasible that Yap1 chooses different transcription factors as a partner on distinct promoters under various stress conditions.

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