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Glutathione peroxidase 3 of *Saccharomyces cerevisiae* regulates the activity of methionine sulfoxide reductase in a redox state-dependent way

Chang Won Kho [a,d], Phil Young Lee [a], Kwang-Hee Bae [a], Sayeon Cho [b], Zee-Won Lee [c], Byoung Chul Park [a], Seongman Kang [d], Do Hee Lee [a,*], Sung Goo Park [a,*]

[a] *Proteome Research Laboratory, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-333, Republic of Korea*
[b] *College of Pharmacy, Chung-Ang University, Seoul 156-756, Republic of Korea*
[c] *Glycomics Team, Korea Basic Science Institute, Daejeon 305-333, Republic of Korea*
[d] *School of Biotechnology, Korea University, Seoul 136-701, Republic of Korea*

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Abstract

Glutathione peroxidase (Gpx) is one of the most important anti-oxidant enzymes in yeast. Gpx3 is a ubiquitously expressed isoform that modulates the activities of redox-sensitive thiol proteins, particularly those involved in signal transduction pathways and protein translocation. In order to search for the interaction partners of Gpx3, we carried out immunoprecipitation/2-dimensional gel electrophoresis (IP-2DE), MALDI-TOF mass spectrometry, and a pull down assay. We found that Mxr1, a peptide methionine sulfoxide reductase, interacts with Gpx3. By reducing methionine sulfoxide to methionine, Mxr1 reverses the inactivation of proteins caused by the oxidation of critical methionine residues. Gpx3 can interact with Mxr1 through the formation of an intermolecular disulfide bond. When oxidative stress is induced by H_2O_2, this interaction is compromised and the free Mxr1 then repairs the oxidized proteins. Our findings imply that this interaction links redox sensing machinery of Gpx3 to protein repair activity of Mxr1. Based on these results, we propose that Gpx3 functions as a redox-dependent exquisite regulator of the protein repair activity of Mxr1.

Keywords: Gpx3; Mxr1; Oxidative stress; ROS; Disulfide bond

Oxidative stress can be defined within the context of a subtly changed redox status [1]. From this point of view, oxidative stress occurs in a cell when an imbalance exists between generation and elimination of reactive oxygen species (ROS) [2]. Oxidative stress is damaging to all cellular constituents and is believed to play a causal role in many degenerative diseases. Accordingly, the cell possesses a variety of defense mechanisms to cope with oxidative stress. *Saccharomyces cerevisiae*, widely used for studying cellular responses to ROS, contains three glutathione peroxidase (Gpx) proteins, i.e., Gpx1 (YKL026C), Gpx2 (YBR244W), and Gpx3 (YIR037W) [3]. The nucleotide sequence of gpx3 was reported and was also referred to as the HYR1 (hydrogen peroxide resistance) gene (unpublished results from SGD yeast genome database). The basal mRNA level of Gpx3 is constitutively higher than that of two other gpx genes. The *gpx3*Δ mutant is hypersensitive to peroxides whereas disruption of gpx1 or gpx2 does not show any obvious phenotype with respect to tolerance to oxidative stress [3]. Therefore, the *gpx3* gene product may be a major Gpx in *S. cerevisiae*. Recently, Delaunay et al. identified a novel role for yeast Gpx3 as a sensor and transducer of the stress response to hydrogen peroxide [4]. This activity occurs via Gpx3-mediated oxidation of the Yap1 transcription factor; the proposed model involves the sensing of high H_2O_2 concentration at the cys36 residue of

* Corresponding authors. Fax: +82 42 860 4593.
E-mail addresses: do_lee@kribb.re.kr (D.H. Lee), sgpark@kribb.re.kr (S.G. Park).

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Gpx3, which forms a disulfide-linked intermolecular complex with cys598 in Yap1. After rearrangement of the disulfide bond, it resolves to yield Yap1 (cys303–cys598) and re-reduced Gpx3. Yap1 may alternatively be activated by thiol reactive chemicals via a Gpx3-independent pathway. It was shown that Gpx3-dependent resistance to H_2O_2 is due to its redox signaling function and not to its peroxidase activity, as suggested in [4].

Protein oxidation can lead to conformation changes and, in some cases, loss of function. Notably, the oxidation of critical amino acids in essential proteins could be a lethal event. Amino acids readily prone to ROS oxidation include cysteine, histidine, tryptophan, tyrosine, and methionine, the latter being the most sensitive [5]. Most cells contain methionine sulfoxide reductase, which catalyzes the thioredoxin-dependent reduction of protein methionine sulfoxide residues back to methionine. The oxidation of methionine residues of some proteins may lead to either activation or inactivation of their biological activities [6], whereas the oxidation of one or more methionine residues in other proteins may have little or no effect on biological functions. Yeast peptide methionine sulfoxide reductase, Mxr1, may serve to repair oxidative damage in some proteins and play an important role in the regulation of enzyme activities by facilitating the inter-conversion of specific methionine residues of these proteins between oxidized and reduced forms [7], in the regulation of various plasma proteinase activities and hormones in the calmodulin-dependent activation of plasma membrane Ca-ATPase [8], and in the modulation of potassium channel function [9]. Mxr1 might also serve as an anti-oxidant enzyme to protect proteins from oxidative damage by ROS [10]. A yeast null mutant of *mxr1* was shown to accumulate both free and protein-bound methionine sulfoxide, and its growth was severely inhibited by H_2O_2 treatment [6].

Interestingly, one of the candidate proteins we identified in this study as an interaction partner of Gpx3 under oxidative stress conditions is Mxr1. From a series of experiments, we have obtained evidence that Gpx3 of *S. cerevisiae*, a redox sensor and ROS scavenger, is also involved in the regulation of the protein repairing activity of Mxr1.

Materials and methods

Yeast strains and growth conditions. *Saccharomyces cerevisiae* strain YPH499 (*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*) and the isogenic derivatives were used in all experiments. Cells were grown at 30 °C in YPD (1% yeast extracts, 2% bactopeptone, and 2% glucose), SD media (0.17% yeast nitrogen base without amino acids, 5% ammonium sulfate, 2% glucose, 0.03% adenine hemisulfate, and appropriate amino acids and bases) or galactose induction media (YPD or SD media containing 2% galactose, 1% raffinose, and 0.03% adenine hemisulfate).

DNA constructs. DNA fragments encoding *gpx3* and *mxr1* ORF were amplified from the *S. cerevisiae* genomic DNA library. The N-terminal GST-Gpx3 construct (pGEX-6P-1-Gpx3) was generated by inserting *gpx3* between the *Bam*HI and *Sal*I sites of pGEX-6P-1. The C-terminal Mxr1-His fusion construct (pET-21a-Mxr1) was generated by inserting *mxr1* between the *Bam*HI and *Not*I sites of pET-21a(+). pESC-LEU-Myc-Gpx3, pESC-LEU-Mxr1-flag, and pYES2-NTC-His-Mxr1 were constructed by subcloning *gpx3* into the *Bam*HI and *Sal*I sites of pESC-LEU and *mxr1* into the *Eco*RI and *Not*I sites of pESC-LEU and pYES2-NTC. The Gpx3 and Mxr1 mutants carrying cysteine to serine substitution were created using a two-step PCR amplification method. Gpx3 or Mxr1 cysteine mutants were constructed by replacing the *Bam*HI/*Sal*I fragment of pGEX6p-1-GST-Gpx3 with that of pESC-LEU-Myc-Gpx3 or replacing the *Eco*RI/*Not*I fragment of pESC-LEU-Mxr1-flag with that of pYES2-NTC-His-Mxr1. A null mutant of the *gpx3* gene was constructed using a PCR-based gene deletion strategy [11,12].

Preparation of cell extracts for monitoring of Gpx3 redox state. For the analysis of the redox state of Gpx3 *in vivo*, cultures were stopped by addition of TCA (20% final) and then lysed by acid lysis [13]. Precipitated proteins were solubilized in the presence of either 50 mM NEM (*N*-ethylmaleimide) or 10 mM AMS (4-acetamido-4′-maleimidylstibene-2,2′-disulfonic acid). Proteins were separated by non-reducing or reducing 12% SDS–PAGE and analyzed by immunoblotting with an anti-Myc antibody (IGTherapy).

Isolation and identification of Gpx3-interacting proteins. To study the interacting partners of Gpx3, IP-2DE, pull-down, and redox-2DE using Gpx3 and Gpx3 cys mutants were carried out. For the analysis of Gpx3 interactomes *in vitro*, *Escherichia coli* strain BL21 (DE3) carrying pGEX6P-1-GST-Gpx3 was grown at 37 °C. *E. coli* cells were suspended in 1× lysis buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 5 mM DTT, 1.5% *N*-laurylsarcosine, and protease inhibitors) and lysed by three freeze–thaw cycles followed by sonication. Cell lysates, adjusted to 2% Triton X-100, were incubated with glutathione agarose beads and then washed on ice. Mxr1-His was purified by the same procedure using Ni–NTA–agarose beads.

For the preparation of yeast cell extracts, cells were cultured until the mid-late exponential phase in YPD medium. Cells were resuspended in lysis buffer (50 mM Tris, pH 8.0, 1 mM PMSF, 100 mM NaCl, 1 mM EDTA, and protease inhibitors) and disrupted with glass beads (0.4–0.6 mm diameter, Sigma). For the GST pull-down assay, 1 mg yeast cell extracts were incubated with 20 μg of GST fusion protein bound to glutathione–Sepharose beads at 4 °C for 4 h in 1 ml of the STET binding buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% Triton X-100, and protease inhibitors). After washing the beads with STET binding buffer, the pulled-down complexes were separated by SDS–PAGE. For the detailed analysis of Gpx3 interactomes *in vitro*, *gpx3*Δ mutants carrying Myc-Gpx3, Myc-Gpx3^{C36S}, or Myc-Gpx3^{C82S} were grown at 30 °C in SD medium supplemented with 2% glucose, and then induced for 12 h with induction media (SD media containing 2% galactose and 1% raffinose). Yeast cells treated with or without H_2O_2 were suspended in lysis buffer for 1 h on ice and disrupted by glass beads. The clarified cell lysates were immunoprecipitated with monoclonal anti-Myc antibody immobilized on protein A&G agarose beads. The eluted samples were analyzed by 2-DE.

Co-immunoprecipitation. Yeast cells were transformed with both pESC-leu-Myc-Gpx3 and pESC-leu-flag-Mxr1 or with pESC-leu-Myc-Gpx3 for immunoprecipitation analysis. The expression of Myc-Gpx3 and flag-Mxr1 was induced in galactose induction media. After treatment with H_2O_2, the cells were lysed in lysis buffer for 1 h on ice and disrupted with glass beads. Twenty microliters of the cell lysates was used for the Western blotting analysis. For co-immunoprecipitation, 1 ml of the cell lysates was incubated with anti-Flag M2 affinity gel (Sigma) at 4 °C overnight. For immunoprecipitation, 1 ml of the cell lysates was incubated with mouse anti-Myc monoclonal antibody (IGtherapy) at 4 °C for 4 h, and 40 μl of protein A/G–agarose beads (Calbiochem) was added to the reaction mixture. The agarose beads were then washed three times with lysis buffer and resuspended in 1× SDS–PAGE sampling buffer for immunoblot analysis.

Pull-down of his-tagged proteins. Both pESC-leu-Myc-Gpx3 and pYES2-NTC-His-Mxr1 were introduced into yeast. The expression of Myc-Gpx3 and His-Mxr1 was induced in galactose induction media. After treatment with H_2O_2, cells were lysed in Ni–NTA lysis buffer (20 mM Tris–HCl, pH 8.0, 50 mM NaH_2PO_4, 500 mM NaCl, 1% Triton X-100, 1 mM PMSF, and a mixture of protease inhibitors) and incubated for 1 h on ice and then disrupted with glass beads. Cell lysates containing His-Mxr1 and

Myc-Gpx3 were mixed with Ni–NTA–agarose beads (Peptron Inc.), incubated for 3 h at 4 °C, and washed with washing buffer to remove nonspecifically bound proteins. Bound proteins were eluted with 1× SDS–PAGE sampling buffer containing 300 mM imidazole and then resolved by SDS–PAGE followed by immunoblotting with an anti-Myc antibody.

Immunoblot analysis of carbonylated proteins. For the detection of oxidized proteins in cell [14–16], the yeast cell extracts were divided into two portions. For derivatization, 5 μl of 12% (w/v) SDS was mixed with 5 μl (20 μg) of cell extracts followed by 10 μl of 1× solution of DNPH (2,4-dinitrophenylhydrazine) reagent (Oxyblot™ Protein Oxidation Detection Kit; Chemicon). The contents were mixed by vortexing and then incubated for 15 min at ambient temperature with periodic agitation. The reaction was stopped by the addition of 7.5 μl of the neutralization solution. After electrophoresis and immunoblotting, proteins containing DNP-derivatized carbonyls were detected by immunostaining the membrane utilizing the Oxyblot™ Protein Oxidation Detection System Kit followed by incubation for 1 min in a 1:1 (v/v) mixture of chemiluminescent reagent and stabilized peroxide solution (Pierce).

Determination of met (O) levels in the cell extract. Yeast cells were grown aerobically in a synthetic complete medium at 30 °C with or without 1 mM H_2O_2 for 30 min. When the cell density reached the early stationary phase ($OD_{600} = \sim 1.5$), cells were collected and washed five times with PBS before their disruption in buffer B (6 M guanidine chloride, 500 mM potassium-phosphate, pH 2.5) containing glass beads. After centrifugation at 20,000*g* for 20 min, the supernatants were passed through microconcentrators (microcon YM-3, Amicon). The retained material was kept for a protein bound amino acid analysis, after extensive washing with sterilized water. The protein moiety of each preparation was subjected to Cobra cleavage [17]. CNBr cleaves peptide bonds on the carboxyl side of met (but not of such bonds involving met (O)) to yield homoserine [18]. One hundred microliters of CNBr solution was added to the vial, which was capped and incubated for 1 h at 70 °C in a hood [17]. After lyophilization, the dried hydrolysates were dissolved in water and subjected to hydrolysis with 6 N HCl for 20 h at 110 °C with or without 5 mM DTT. Hydrogen chloride hydrolysis and amino acid analysis were carried out on samples with and without CNBr treatment as described [19].

Measurement of intracellular ROS levels. To measure the intracellular levels of ROS, yeast cells were grown in YPD or SD medium until OD_{600} reached 1.5. After treatment with H_2O_2 for 10 min, the cells were collected and suspended in fresh media. Cells were subsequently reacted with 20 μM 5-(and -6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, and acetyl ester (CM-H_2DCFDA), and incubated for 1 h at room temperature. The cells were immediately analyzed by a laser-scanning confocal microscope (LSM 410, Carl Zeiss) with excitation and emission settings of 488 and 515 nm, respectively. About 50 cells were randomly selected from three separate experiments and relative DCF fluorescence intensities of the treated cells were compared with those of unstimulated control cells [20,21].

Determination of tolerance against various oxidants. Yeast cells were cultured to the mid-late-exponential phase either in YEPG medium (1% yeast extract, 2% peptone, and 2% galactose) or yeast nitrogen base medium supplemented with the appropriate amino acids or bases. For spotting experiments, the number of yeast cells was adjusted and then spotted on YEPD agar supplemented with H_2O_2 or met (O) as indicated. In the case of the liquid test, yeast cells were grown in YEPG medium, and cell numbers were adjusted with fresh YPG. Growth was followed by measuring the OD_{600}. During the late-log growth phase (OD_{600} of 0.6), cultures were split into two subcultures, one of which was treated with oxidants (H_2O_2, final concentration 1 mM) [22].

Results

Constructions of gpx3 deletion mutants and purification of recombinant Gpx3

To investigate the exact function(s) of Gpx3 with respect to the oxidative stress responses of *S. cerevisiae*, we have analyzed the interaction partners of Gpx3. To exclude the effect of endogenous Gpx3 in the experiments, we first constructed the *gpx3*-deleted mutant of *S. cerevisiae*. We confirmed that the expression of Gpx3 was completely abolished in the *gpx3*Δ mutant, which also showed a H_2O_2-sensitive phenotype (Fig. 1A and B). We also constructed a mutant of Gpx3 in which an internal cysteine at the active site was substituted with serine. Using this protein, we effectively acquired the interacting proteins in yeast when they formed the mixed-disulfide intermediates with the cys residue mutant Gpx3 (see below). For *in vitro* experiments, recombinant Gpx3 and Gpx3 cys mutants were expressed and purified from *E. coli*. In Gpx3 mutants carrying substitutions of cysteines ($Gpx3^{C36S}$, $Gpx3^{C82S}$, and $Gpx3^{C36/82S}$), the oxidized form of Gpx3 was almost completely disappeared when they were treated with H_2O_2 (Fig. 1C). In contrast, the oxidized form appeared in recombinant Gpx3. The peroxidase activity of recombinant Gpx3 used in these experiments was evaluated by measuring the oxidation of NADPH. As already known, Gpx3 was found to be more active with the thioredoxin system than with the GSH system as a reducing source (data not shown) [4,23].

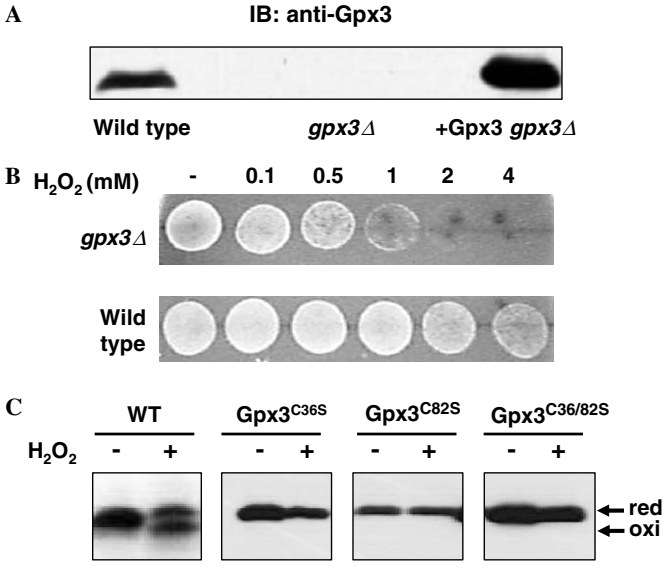
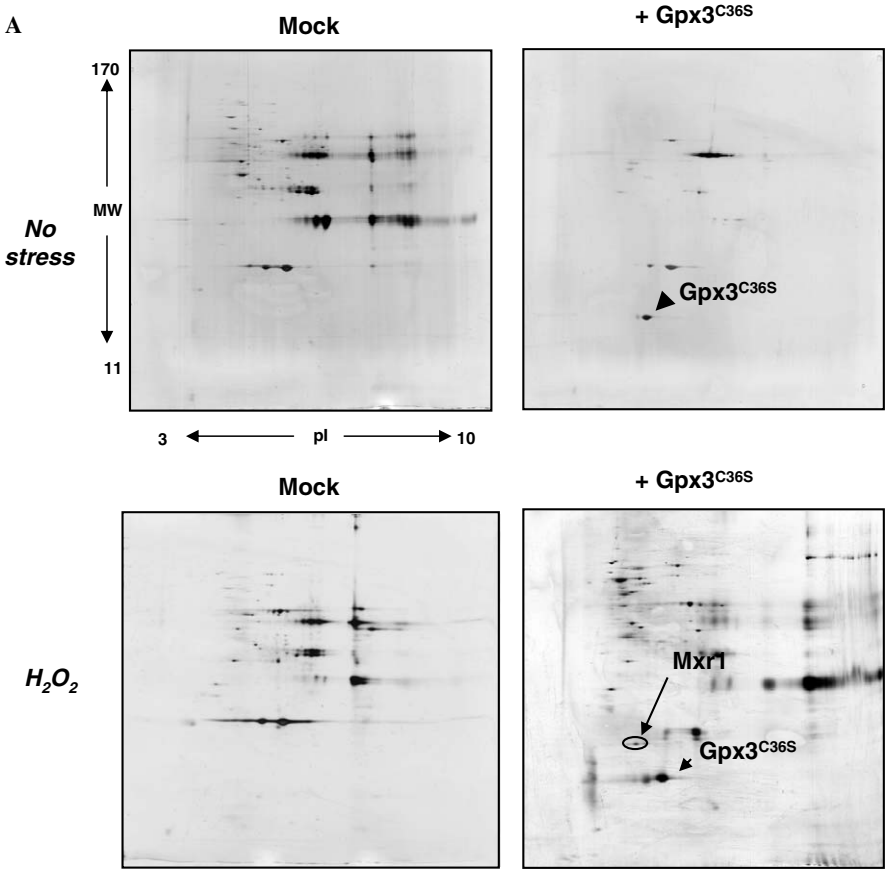


Fig. 1. Construction of *gpx3*Δ mutant and expression of recombinant Gpx3 protein. (A) Confirmation of *gpx3* gene deletion in the *gpx3*Δ mutant. Yeast cell proteins were separated by 12% SDS–PAGE and analyzed by immunoblotting with an anti-Gpx3 antibody. (B) Measurement of resistance of *gpx3*Δ mutant against the peroxide treatment. Yeasts were cultured to mid- to late exponential phase ($OD_{600} = \sim 1.0$) in liquid YPD medium. For spotting experiments, the cultures were diluted to OD_{600} of 0.001 and the samples from each dilution were spotted on YPD agar supplemented with H_2O_2 (0.1–4 mM). Photographs were taken after 4 days of incubation at 30 °C. (C) *In vivo* analysis of the redox state of Gpx3 and Gpx3 Cys mutants. NEM-blocked cell extracts from gpx3Δ over-expressing Gpx3-Myc, $Gpx3^{C36S}$-Myc, $Gpx3^{C82S}$-Myc, or $Gpx3^{C36/82S}$-Myc were treated with or without 0.4 mM H_2O_2 for 5 min and subjected to non-reducing SDS–PAGE followed by immunoblotting with an anti-Myc antibody. Data are representative of three separate experiments.

Gpx3 interacts with Mxr1

For the preliminary experiment, the whole cell extract from the *gpx3*Δ mutant was incubated with GST-Gpx3 fusion protein bound to glutathione–agarose beads. After extensive washings, bound proteins were eluted and resolved by SDS–PAGE. Several protein bands that were absent in the control lanes were detected by colloidal Coomassie blue staining (data not shown). For the further analysis of the Gpx3 interactomes, cell lysate from H_2O_2-treated *gpx3*Δ mutant over-expressing Myc-Gpx3 cys mutant ($Gpx3^{C36S}$) was subjected to the immunoprecipitation with polyclonal anti-Myc antibody immobilized on protein A&G agarose beads. During the redox pathway, a specific protein that has free thiol group forms mixed-disulfide intermediates with the target proteins. Then the specific protein itself forms an internal disulfide bridge, thus releasing the reduced target proteins, which makes it difficult to isolate the target proteins with the wild-type protein. This intermediate state could be stabilized when the specific protein lacks the second cysteine residue [24]. For this reason, we used $Gpx3^{C36S}$ lacking the second cysteine residue as the bait and isolated the interacting proteins more efficiently. The eluted samples were separated by 2DE and the spots showing quantitative differences were identified using MALDI-TOF mass spectrometry (Fig. 2A). Among the identified Gpx3 interacting proteins, we selected peptide methionine sulfoxide reductase 1 (Mxr1 encoded by ORF YER042w) for further study (Fig. 2B).

To examine whether Gpx3 interacts with Mxr1 *in vitro*, recombinant His-tagged Mxr1 was expressed and purified from *E. coli*. The purified 23 kDa His-tagged Mxr1 was capable of reducing the free met (O) to met in the presence of the thioredoxin system [25] or DTT as a reducing agent [26], demonstrating that the recombinant protein was functionally active (data not shown). We next tested whether



B

Description	Accession No.	Mr/pI	Coverage (%)
Peptide methionine sulfoxide reductase (Protein-methionine-S-oxide reductase) Mxr1	**P40029**	**21141 /6.5**	**35 ~ 40**

Fig. 2. Isolation and identification of Gpx3-interacting proteins. (A) Silver-stained 2-DE image of proteins immunoprecipitated with polyclonal anti-Myc antibody (IGtherapy) immobilized on protein A&G agarose beads after H_2O_2 treatment of *gpx3*Δ mutant strain over-expressing $Gpx3^{C36S}$ and mock control. The $Gpx3^{C36S}$ is indicated by the arrow. The spot indicated by the circle shows quantitative differences between normal and stress conditions. Data are representative of three separate experiments. (B) For protein identification, the spots indicated by the circle were excised and subjected to trypsin digestion. Subsequent peptide mass fingerprinting analysis revealed that this spot was yeast Mxr1.

Gpx3 interacts with Mxr1 in yeast cells. After treatment with or without H_2O_2, cell lysates of the wild-type over-expressing Myc-Gpx3, Myc-Gpx3^{C36S}, or Myc-Gpx3^{C82S} were immunoprecipitated with anti-Myc antibody. Under the normal state, endogenous Mxr1 associated with the wild-type Gpx3 but not with Gpx3^{C82S} mutant and their interaction was abolished by H_2O_2 treatment (Fig. 3A). By contrast, Gpx3^{C36S} interacted more efficiently with Mxr1 and H_2O_2 treatment actually enhanced their interaction (Fig. 3A). This result implies that cys82 of Gpx3 is involved in the interaction with Mxr1 and that cys36 in the formation of internal disulfide bridge. To prove that Mxr1 interacts with endogenous Gpx3 reciprocally, we treated the wild-type strain carrying Mxr1-flag with or without H_2O_2 and then isolated Mrx1 by immunoprecipitation. After IP, the eluted proteins were separated by 2DE and analyzed by immunoblotting with an anti-Gpx3 antibody. As shown in Fig. 3B, endogenous Gpx3 interacted with Mxr1 under the normal state. However, we could not detect Gpx3 after H_2O_2 treatment. Together these results indicate that wild-type Gpx3, presumably via cys82, interacts with Mxr1 and oxidative stress impairs such interaction.

Gpx3 interacts with Mxr1 through disulfide bond

Above observation that Gpx3^{C82S} could not interact with Mxr1 suggests that the cys82 of Gpx3 mediates the interaction with Mxr1, likely through the formation of a disulfide

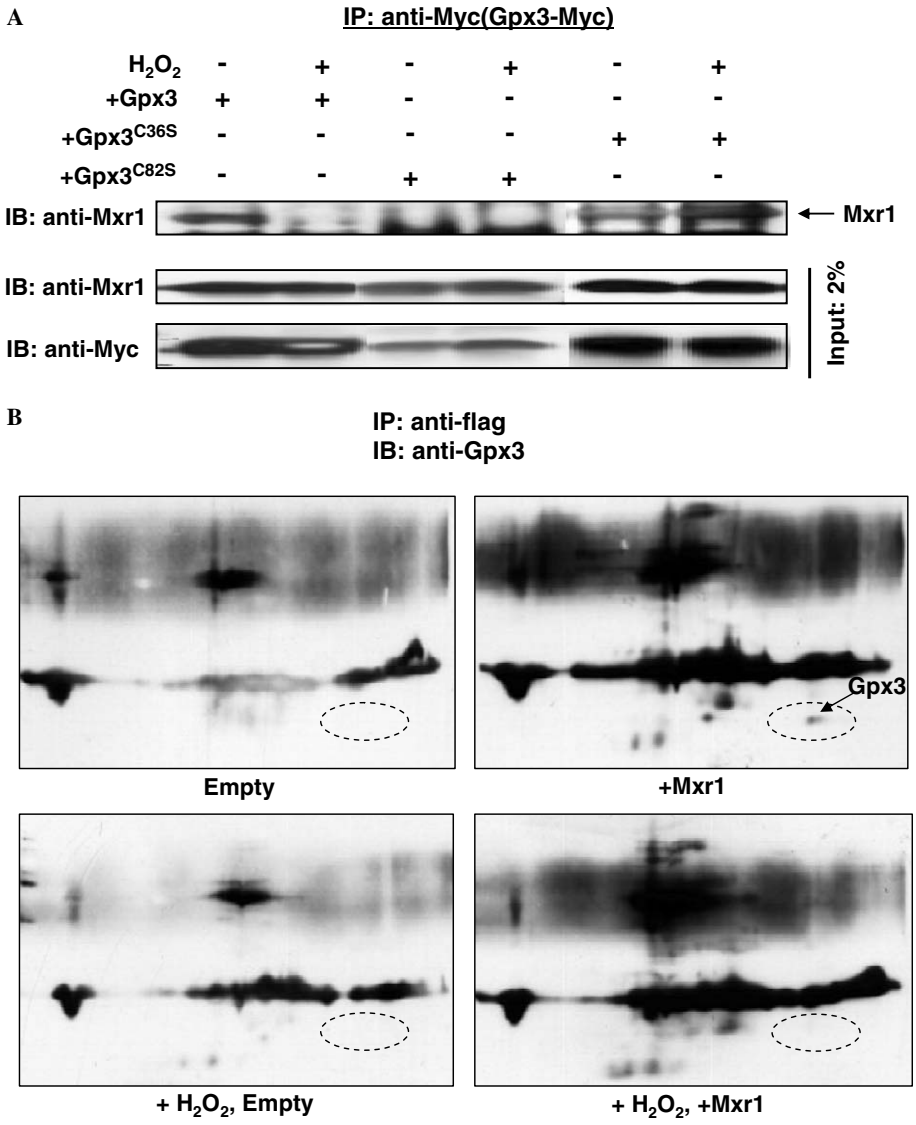


Fig. 3. Gpx3 associates with Mxr1 *in vivo*. (A) Interaction between Gpx3 and Mxr1 is severely compromised by oxidative stress. Gpx3 was isolated with an anti-Myc antibody (IP: anti-Myc) from extracts of the *gpx3*Δ strains over-expressing Myc-Gpx3 or Myc-Gpx3^{C82S} treated with or without 0.4 mM H_2O_2 for 5 min. The immunoprecipitated proteins were probed with the polyclonal anti-Mxr1 antibody. (B) Mxr1 was isolated with an anti-flag beads (IP: anti-flag) from extracts of the wild-type over-expressing flag-Mxr1 treated with or without 0.4 mM H_2O_2 for 5 min. The immunoprecipitated proteins were analyzed by 2-DE and probed with the polyclonal anti-Gpx3 antibody. The Gpx3 spot on the 2-D gels is indicated by arrow. Production and purification of polyclonal antibodies against yeast Gpx3 and Mxr1 were accomplished by Aprogen (Daejeon, S. Korea). The blots shown are representative of three independent experiments.

bond. For further investigation, we then examined the formation of intermolecular disulfide bond between Gpx3 and Mxr1 *in vivo* and the effect of redox condition on this process. First, we observed that Gpx3 interacted with Mxr1 under normal state and this interaction was greatly reduced when cells were treated with H_2O_2 or DTT (Fig. 4A). $Gpx3^{C36S}$ also strongly interacted with Mxr1 under normal and oxidative conditions whereas the treatment of DTT reduced their interaction (Fig. 4B). Next, to determine the Gpx3 interaction site of Mxr1, we pulled-down Mxr1 from cell extracts of *gpx3*Δ strains carrying both Myc-$Gpx3^{C36S}$ and His-Mxr1 cys mutants ($Mxr1^{C25S}$ or $Mxr1^{C176S}$) after H_2O_2 treatment. For these experiments, we used $Gpx3^{C36S}$ because this mutant shows a stronger interaction with Mxr1 than the wild-type Gpx3. When cys176 of Mxr1, one of the putative active sites, was substituted with serine, the interaction between $Gpx3^{C36S}$ and Mxr1 was severely decreased; whereas the substitution of cys25 did not affect the interaction between these two proteins (Fig. 4C). Together with above results, this observation suggests that cys82 of Gpx3 may associate with cys176 of Mxr1 via an intermolecular disulfide bond.

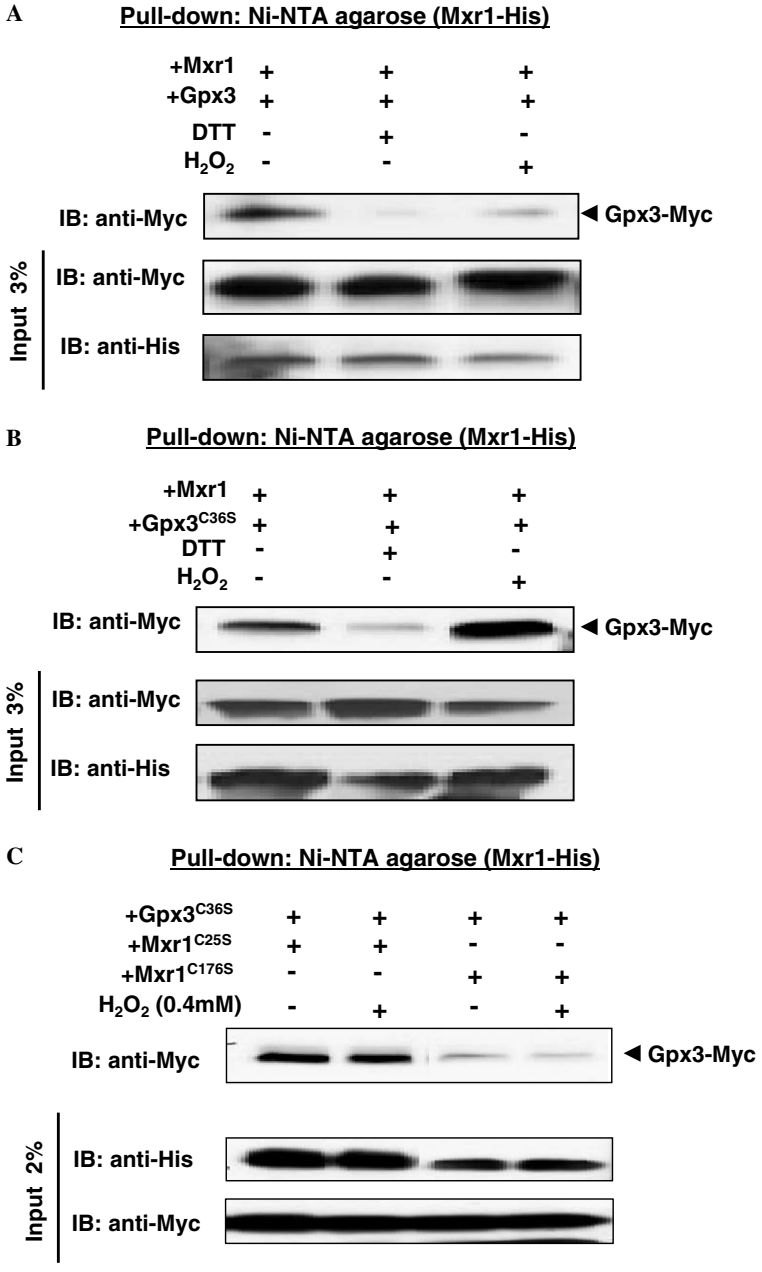


Fig. 4. Analysis of the interaction between Gpx3 and Mxr1 cys mutants *in vivo*. After the treatment with 1 mM H_2O_2 or 4 mM DTT for 5 min, Mxr1 was pulled-down from the extracts of *gpx3*Δ strains over-expressing His-Mxr1 and Myc-Gpx3 (A) or His-Mxr1 and Myc-$Gpx3^{C36S}$ (B) using Ni–NTA–agarose beads. The presence of Gpx3 was probed with an anti-Myc antibody. (C) His-Mxr1 cys mutants were pulled-down from the extracts of *gpx3*Δ strain over-expressing His-Mxr1 and Myc-$Gpx3^{C36S}$ using Ni–NTA–agarose beads. The presence of Gpx3 was probed with an anti-Myc antibody. The blots shown are representative of three independent experiments.

We also examined the *in vitro* formation of intermolecular disulfide bond between Gpx3 and Mxr1 using non-reducing SDS–PAGE and Western blotting. After H_2O_2 treatment of a mixture containing Gpx3 and Mxr1, we observed that several faint, higher molecular weight bands appeared (data not shown). Their absence under the reducing condition further indicated a probable inter- or intramolecular disulfide linkage between Gpx3 and Mxr1. Indeed, a MALDI-TOF MS analysis of these higher molecular weight bands demonstrated that they were a complex of Gpx3 and Mxr1 (data not shown).

Intracellular ROS level is affected by the transducer function of Gpx3

To understand whether intracellular ROS level is influenced by the effect of Gpx3 on Mxr1 activity, we examined the changes of intracellular ROS level in the wild-type, *gpx3*Δ mutant, and *gpx3*Δ mutants over-expressing Gpx3, $Gpx3^{C36S}$, or $Gpx3^{C82S}$ (Fig. 5). Both under normal and oxidative stress conditions, the intracellular ROS level in *gpx3*Δ mutant was higher than that of the wild-type, but *gpx3*Δ mutant strain over-expressing Gpx3 showed a ROS level comparable to that of the wild-type (Fig. 5). Interestingly, in *gpx3*Δ mutant over-expressing $Gpx3^{C36S}$, which lacks peroxidase activity, the ROS level was greater than that of the *gpx3*Δ mutant whereas in the *gpx3*Δ mutant over-expressing $Gpx3^{C82S}$, which also lacks peroxidase activity, the ROS level was similar to that of the *gpx3*Δ mutant strain over-expressing Gpx3 (Fig. 5). These results demonstrate that the key factor to regulate intracellular ROS level may not be the peroxidase activity of Gpx3 but other functions of Gpx3, such as Yap1 activation through cys36 [4,27].

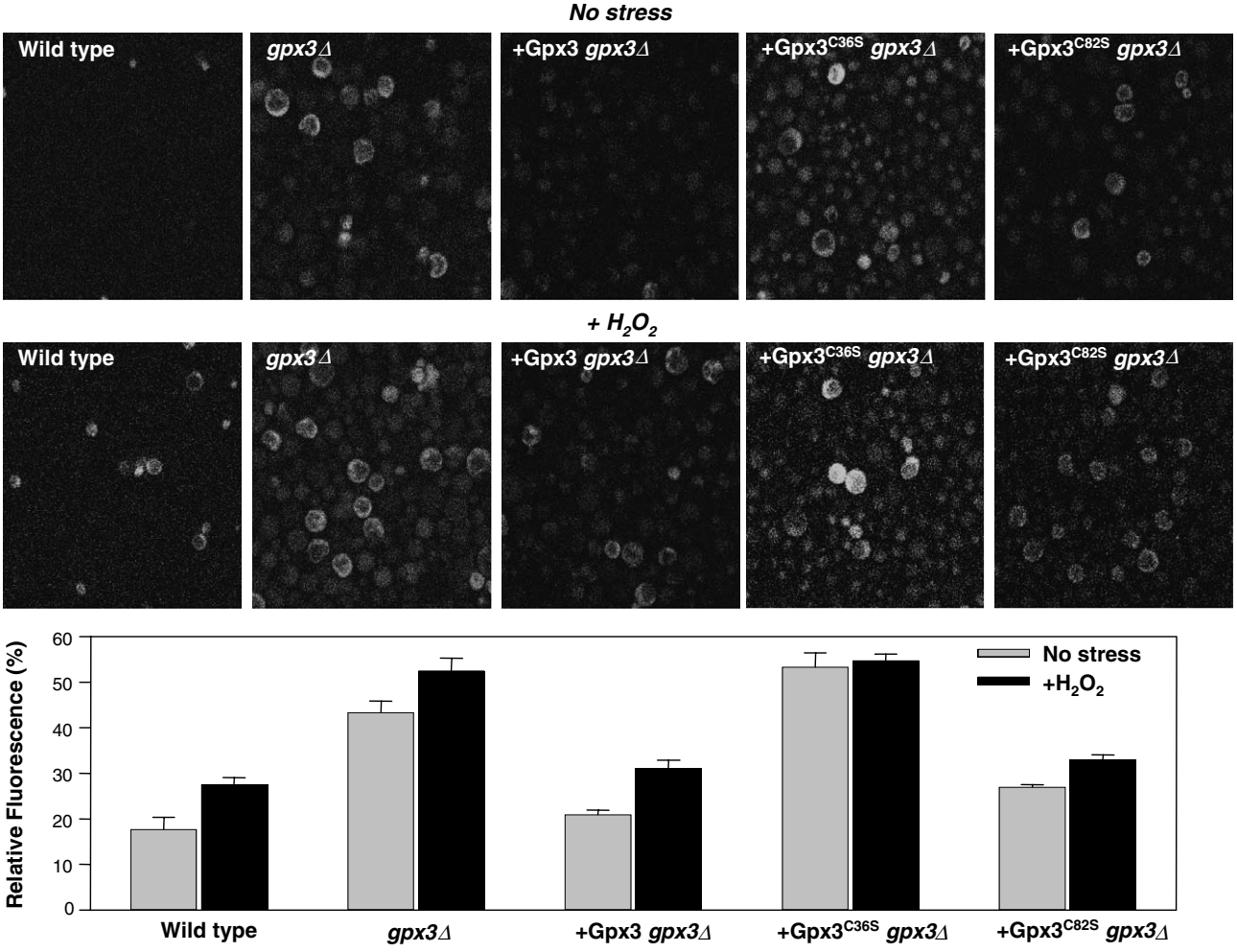


Fig. 5. Changes of intracellular ROS levels in the wild-type, *gpx3*Δ mutant, and *gpx3*Δ mutant overexpressing Gpx3, $Gpx3^{C36S}$, or $Gpx3^{C82S}$. Yeast strains were grown in YPD or SD medium until OD_{600} reached ~1.5. They were then treated with or without H_2O_2 for 10 min and subsequently with 5 μM H2DCFDA for 5 min. DCF images were obtained using a confocal microscope. About 50 cells were randomly selected from three separate experiments and relative DCF fluorescence intensities of treated cells were compared with those of control cells. Fluorescence from the cells untreated with DCF was used as the basal for the relative intensity. The statistical differences were defined as $p < 0.01$ by Student's *t* test. Data are representative of three separate experiments.

Protein repair function of Mxr1 is affected by Gpx3

To determine the effect of Gpx3 on protein oxidation, we analyzed patterns of the oxidized proteins in the wild-type and *gpx3*Δ strain over-expressing Gpx3, or Gpx3 cys mutants, using an Oxyblot™ Protein Oxidation Detection Kit (Fig. 6A). Surprisingly, the *gpx3*Δ strain and *gpx3*Δ strain over-expressing $Gpx3^{C36S}$ (Fig. 6A; lanes 2 and 4) showed rather increased level of oxidative modification of proteins relative to the wild-type whereas the *gpx3*Δ strains over-expressing Gpx3 or $Gpx3^{C82S}$ demonstrated markedly decreased level of modification (Fig. 6A; lanes 3 and 5). The wild-type over-expressing Mxr1 and the *gpx3*Δ strain over-expressing Mxr1 also showed significantly reduced oxidative modification of proteins (Fig. 6A; lanes 6 and 7). These data strongly suggest that the protein repair function of Mxr1 is affected by Gpx3. The dramatic increase of oxidatively modified proteins in the *gpx3*Δ strain over-expressing $Gpx3^{C36S}$ may be partially due to the preferential capturing of the active Mxr1 by $Gpx3^{C36S}$. Cys36 of Gpx3, the active site for peroxidase activity, is required for hydroperoxide reduction and Yap1 activation [4]. In $Gpx3^{C36S}$, both hydroperoxide reduction activity and Yap1 activation function are likely compromised. However, $Gpx3^{C36S}$ remains to strongly bind to Mxr1 and, as a result, causes a dramatic increase of oxidized proteins in cells. In contrast, $Gpx3^{C82S}$ does not have a peroxidase activity, but still can activate Yap1 [4]. In addition, $Gpx3^{C82S}$ cannot interact with Mxr1. Therefore, the decrease of oxidized proteins in the strain over-expressing $Gpx3^{C82S}$ can be accounted for by the presence of active Yap1 and Mxr1.

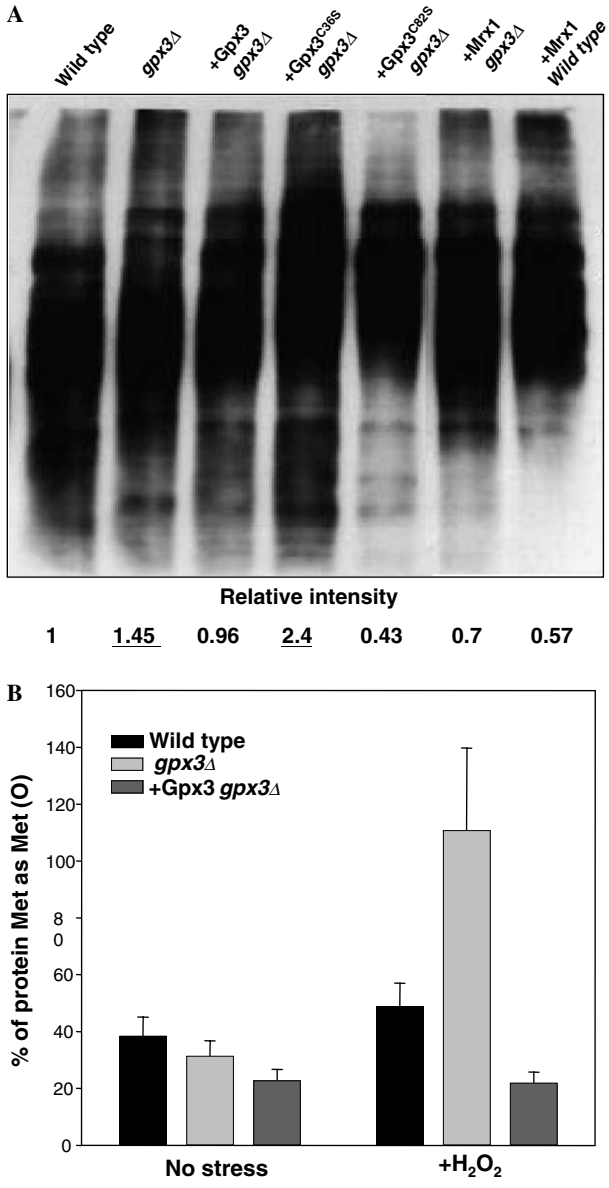


Fig. 6. Analysis of carbonylated proteins under oxidative stress. (A) Proteins containing DNP-derivatized carbonyls were detected by using the Oxyblot™ Protein Oxidation Detection System Kit. The blots shown are representative of three independent experiments. (B) The methionine (O) content was affected by Gpx3. Yeast strains were grown in SD medium with or without 1 mM H_2O_2 until OD_{600} reached ~1.5. Cells were harvested and the amount of protein-bound methionine sulfoxide was measured as described in Materials and methods.

The difference of methionine oxidation between the wild-type and *gpx3*Δ strain was much more pronounced when the content of met (O) was measured under the same conditions. As shown in Fig. 6B, under the normal condition, no changes of protein met (O) level were detected in the wild-type, *gpx3*Δ strain, and *gpx3*Δ strain over-expressing Gpx3. In contrast, a much higher level of protein met (O) was detected in the *gpx3*Δ strain when H_2O_2 was added to the culture medium. As expected, over-expression of Gpx3 restored the level of protein met (O) in the *gpx3*Δ strain after H_2O_2 treatment to the level of protein met (O) of the wild-type.

Cell growth is affected by Gpx3

To evaluate Gpx3 function on the Mxr1 reaction mechanism during yeast cell growth, we monitored the growth of each strain (the wild-type, *gpx3*Δ, *gpx3*Δ over-expressing Gpx3, $Gpx3^{C36S}$, or $Gpx3^{C82S}$) after adding met (O) or H_2O_2 (Fig. 7). Under the normal state, growth of the *gpx3*Δ strain and *gpx3*Δ strain over-expressing $Gpx3^{C36S}$ or $Gpx3^{C82S}$ was slightly delayed when compared to the wild-type and *gpx3*Δ strain over-expressing Gpx3 (Fig. 7). The *gpx3*Δ strain was more sensitive to met (O) than the other strains (Fig. 7, *gpx3*Δ) [28,29]. In addition, the *gpx3*Δ strain over-expressing $Gpx3^{C36S}$ was as sensitive to H_2O_2 and met (O) as the *gpx3*Δ strain (Fig. 7, $+Gpx3^{C36S}$ *gpx3*Δ). The *gpx3*Δ strain over-expressing Gpx3 or $Gpx3^{C82S}$ was more resistant to H_2O_2 than the other Gpx3 mutant strains (Fig. 7, +Gpx3 and $+Gpx3^{C82S}$ *gpx3*Δ). These results again indicate that Gpx3 affects the activity of Mxr1 under the oxidative stress condition, thereby exerting effects on the growth of yeast.

Discussion

All organisms that use oxygen as a terminal electron acceptor in oxidative phosphorylation have to be protected

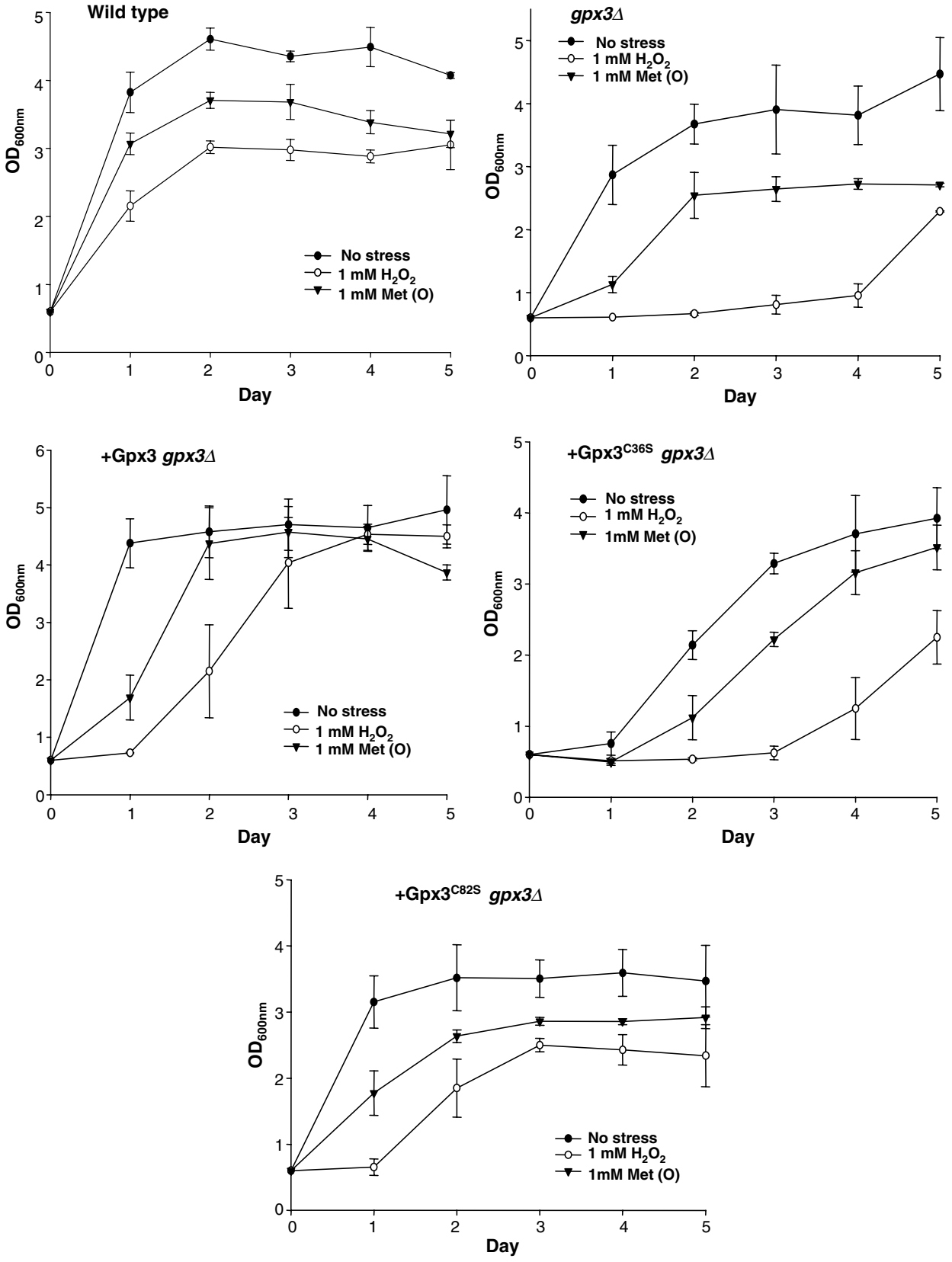


Fig. 7. Growth of yeast strains under oxidative stress condition or in the presence of methionine (O) as sulfur source. Cells grown overnight in YEPG (1% yeast extract, 2% peptone, and 2% galactose) medium were resuspended in fresh medium ($OD_{600} = 0.2$). At the late-log growth phase ($OD_{600} = 0.6$), the cultures were divided into two groups, one of which was treated with 1 mM H_2O_2 or 1 mM methionine (O). Their growth was monitored until they reached the stationary phase. Data are representative of three separate experiments.

from ROS, which damages proteins, DNA, and membrane fatty acids. In order to cope with oxidative stress, cells possess a range of nonenzymatic and enzymatic defense systems including glutathione, thioredoxin, glutaredoxin, superoxide dismutase, and peroxidases [3,30]. A series of protecting systems can be classified into two subsets. A subset of these systems, which includes catalase, peroxidase, and superoxide dismutase, acts by reducing endogenous levels of ROS. Another subset includes enzymes to repair ROS damage (oxidized lipid, DNA, or proteins). In this study, we found that Gpx3, which has a peroxidase activity and redox-sensing and signal transducing activity, is also linked with Mxr1, which repairs damaged proteins.

Gpx3 and Mxr1 exhibit broad substrate specificity and each can reduce different classes of proteins. Our results present convincing evidence for the regulation of Mxr1 by Gpx3. We have revealed that Gpx3 affects the enzymatic activity of Mxr1 under oxidative stress. We have also confirmed that Gpx3 is physically associated with Mxr1. In addition, protein oxidation was decreased in the strains over-expressing Gpx3, $Gpx3^{C82S}$, or Mxr1 when oxidative stress occurs. Although $Gpx3^{C82S}$ lacks peroxidase activity, it still possesses the ability to defense against oxidative stress. *In vivo* analysis of Mxr1 activity on the background of *gpx3*Δ mutants, *gpx3*Δ strains over-expressing Gpx3, showed similar activity to the wild-type. Of the three cysteines in Gpx3, cys36 aligns with the peroxidatic selenocysteine 52 of the bovine enzyme, and both are essential for the peroxidase activity. Interestingly, Delaunay et al. recently demonstrated a second, more important role for Gpx3 in cellular defense against H_2O_2-mediated oxidative stress [4]. Cys36 of Gpx3, instead of forming the intramolecular disulfide, can alternatively form an intermolecular protein disulfide bond with cys598 of Yap1. This Gpx3-S-S-Yap1 intermediate undergoes a subsequent intramolecular thiol-disulfide interchange involving Yap1 cys303. While Gpx3 cys82 is associated with lipid peroxidation [31], we suggest that this cysteine residue also plays an important role in the defense against oxidative stress.

We demonstrated that the Mxr1 activity of *gpx3*Δ strain was severely decreased but recovered to a level comparable to that of the wild-type after treatment of H_2O_2. The level of protein oxidation in the cell from the *gpx3*Δ strain and *gpx3*Δ strain over-expressing $Gpx3^{C36S}$ was significantly increased. However, protein oxidation of the *gpx3*Δ strain over-expressing $Gpx3^{C82S}$ was greatly reduced to a level similar to that of the *gpx3*Δ strain over-expressing Mxr1. Our results suggest a novel functional link between Gpx3 and Mxr1. In cells, cys36 of Gpx3 is the site of peroxide sensing, while cys82 of Gpx3 is able to bind to cys36-SOH, the expected oxidation product of a cysteine residue by hydroperoxides [4]. Cys82 of Gpx3 binds to cys176 of Mxr1 in the normal state, which is switched to cys36 of Gpx3 upon the insult of oxidative stress through a thiol-disulfide exchange reaction. Thereafter, released Mxr1 could repair the oxidized proteins in the cells. Mxr1 is also known to use an intra- or inter-disulfide bond exchange mechanism such as Gpx3. Cys176 of Mxr1 functions as a recycling cysteine during the repairing process [32]. These two recycling cysteines (cys82 of Gpx3 and cys176 of Mxr1) are most likely bridged in the normal state. Upon oxidative stress, this disulfide bond is broken and the protein repair activity of Mxr1 is timely turned on. Therefore, we strongly suggest that cys82 of Gpx3 does not associate with the peroxidase activity, but functions to assist the defense mechanism of Mxr1 against oxidative stress. Such interaction between Gpx3 and Mxr1 under oxidative stress may serve as an important and efficient regulatory link between ROS detoxification enzymes and repairing proteins.

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