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Interactome Analysis of Yeast Glutathione Peroxidase 3

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Oxidative stress damages all cellular constituents, and therefore, cell has to possess various defense mechanisms to cope. *Saccharomyces cerevisiae*, widely used as a model organism for studying cellular responses to oxidative stress, contains three glutathione peroxidase (Gpx) proteins. Among them, Gpx3 plays a major defense role against oxidative stress in *S. cerevisiae*. In this study, in order to identify the new interaction proteins of Gpx3, we carried out two-dimensional gel electrophoresis after immunoprecipitation (IP-2DE), and MALDI-TOF mass spectrometry. The results showed that several proteins including protein disulfide isomerase, glutaredoxin 2, and SSY protein 3 specifically interact with Gpx3. These findings led us to suggest the possibility that Gpx3, known as a redox sensor and ROS scavenger, has another functional role by interacting with several proteins with various cellular functions.

Keywords: Glutathione peroxidase 3, IP-2DE, oxidative stress, ROS

All living organisms suffer from various stresses derived from a number of environmental factors such as oxidants, heat, irons, cytotoxic agents, and environmental toxins. A series of stresses induces oxidative stress to the host organisms. Oxidative stress occurs in a cell when an imbalance exists between generation and removal of reactive oxygen species (ROS) [20]. Oxidative stress is damaging to all cellular constituents, increases with age, and plays a causal role in many degenerative diseases such as cancer [17, 18].

The molecular basis of the response to oxidative stress has been extensively studied in *Saccharomyces cerevisiae* [10, 12, 13]. *S. cerevisiae* contains three glutathione peroxidase (Gpx) proteins, *i.e.*, Gpx1 (YKL026C), Gpx2 (YBR244W), and Gpx3 (YIR037W) [11]. The *gpx3* mutant is hypersensitive to peroxides, whereas disruption of *gpx1* or *gpx2* does not show any obvious phenotypes with respect to the tolerance to oxidative stress [5]. Therefore, the Gpx3 protein is thought to be a major Gpx functioning in scavenging peroxides in *S. cerevisiae*. Recently, Delaunay *et al.* [3] identified a novel role for yeast Gpx3 as a sensor and a transducer of the stress response against hydrogen peroxide. This activity occurs *via* Gpx3-mediated oxidation of the Yap1 transcription factor [2, 15].

Although previous reports suggest that Gpx3 is a major antioxidant enzyme in the detoxification of ROS, the regulation and defense mechanism of Gpx3 in cellular redox homeostasis is still unclear [19]. Therefore, for mining another function of Gpx3, the new interacting partners of Gpx3 were identified by proteomic methods [9]. *S. 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. The Gpx3 was deleted out using a PCR-mediated gene deletion method as previously described [5]. The DNA fragment encoding Gpx3 was amplified from genomic DNA by PCR using a pair of primers covering the entire coding sequence (Forward primer: 5'-CGCGGATCC ATG TCA GAA TCC-3'; Reverse primer: 5'-ACGCGT CGA CCT ATT CCA CCT C-3'). The PCR product was cloned into yeast expression vector pESC-LEU (Stratagene). We also constructed mutants of Gpx3 ($Gpx3^{C36S}$ and $Gpx3^{C82S}$), in which an internal cysteine at the active site was replaced with serine. Using these proteins, we effectively obtained the interacting proteins when they formed the mixed-disulfide intermediates with the cysteine residue mutant Gpx3 (Fig. 1). The *gpx3Δ* mutant carrying Myc-Gpx3, Myc-$Gpx3^{C36S}$, or Myc-$Gpx3^{C82S}$ was 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

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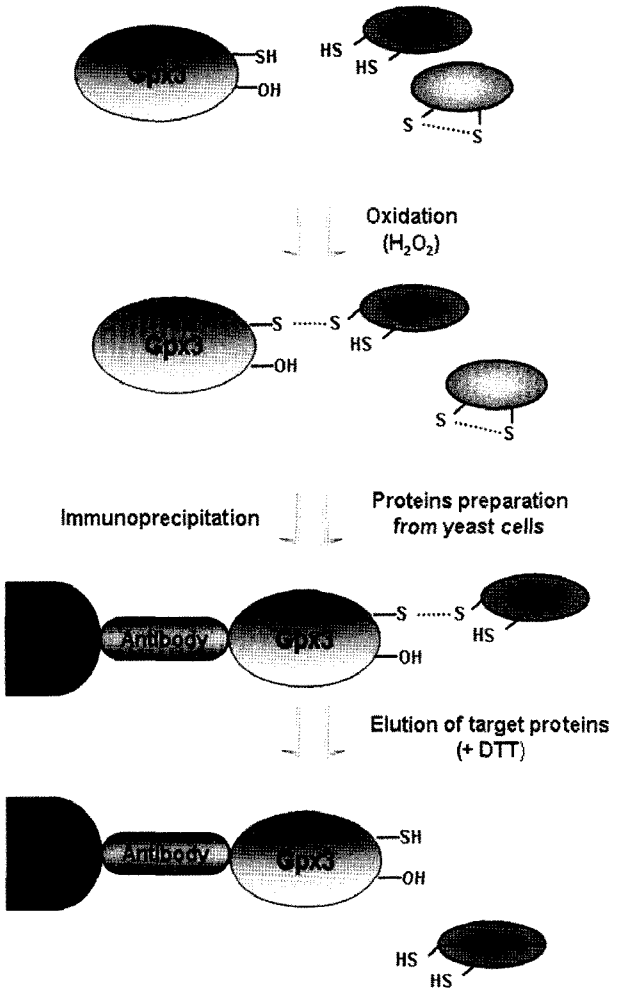

Fig. 1. Schematic experimental flowchart of the analysis of the Gpx3-interacting proteins.
The internal cysteine residue of Gpx3 was substituted with serine and immunobilized on the resin. The mixed-disulfide or nonmixed-disulfide intermediate should be broken by the addition of DTT, thus eluting the target proteins.

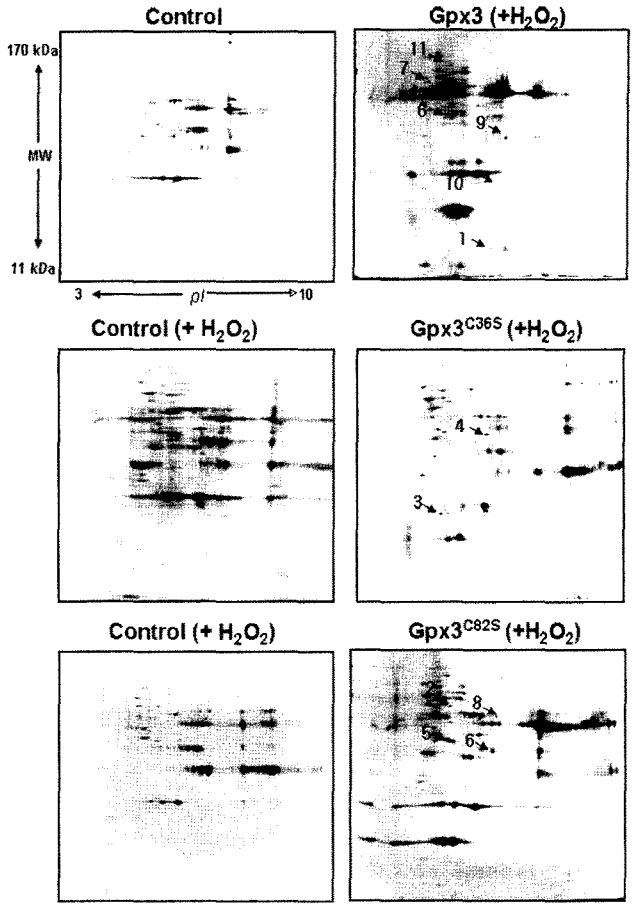

Fig. 2. Silver-stained 2-DE image of proteins immunoprecipitated with polyclonal anti-Myc antibody (IGtherapy) immobilized on protein A&G agarose beads after H_2O_2 treatment of the *gpx3D* mutant strain overexpressing wild-type Myc-Gpx3, Myc-Gpx3^{C36S}, or Myc-Gpx3^{C82S}.
The candidate proteins are indicated by the arrow. Data are representative of three separate experiments.

cells either treated or untreated with H_2O_2 were harvested and resuspended in yeast lysis buffer (50 mM Tris, pH 8.0, 1 mM PMSF, 100 mM NaCl, 1 mM EDTA, and protease inhibitors) for 1 h on ice and disrupted with glass beads at 4°C (0.4–0.6 mm diameter; Sigma) by using a mini-bead beater, and then centrifuged at 13,000 rpm for 45 min at 4°C. The crude proteins from these yeast cells were applied to immunoprecipitation with a monoclonal anti-Myc antibody fixed on protein A&G agarose beads for 2 h at 4°C to ensure the immobilization of the mixed-disulfide or nonmixed-disulfide intermediates. After washing with washing buffer, the trapped proteins on the resin were eluted by elution buffer containing 10 mM DTT. The eluted proteins were applied to two-dimensional electrophoresis (2-DE) to separate the mixed candidate Gpx3 interacting partners. The 2-DE gel was visualized by silver staining, and these images were analyzed with PDQuest-2-DE analysis software (Bio-Rad). Compared with the control (Mock control *vs.* Gpx3, Gpx3^{C36S}, or Gpx3^{C82S} as a bait in normal condition), 11 newly appeared protein spots were detected (Fig. 2). The proteins were excised from the gel and were destained, and in-gel digested with trypsin. The extracted peptides were put through a desalting/concentration step on μZipTipC18 (Millipore Co.) before MALDI-TOF mass spectrometry analysis. Mass spectrometric analyses were performed using a PerSeptive Biosystems MALDI-TOF Voyager DE-RP mass spectrometer operated in the delayed extraction and reflector mode [6–8]. Web-based programs (PEPTIDENT, MS-Fit, Profound, and MASCOT) were used for the database search against the Swiss-Prot database.

Various proteins related with protein folding, metabolism, and transcription were identified (Table 1). As expected, most of them were known to be directly or indirectly involved in stress response. Peptide methionine sulfoxide reductase [9], glutamine synthetase [14], and Yap1-binding proteins [3] were already reported as the interacting proteins

Table 1. Eleven candidate interacting protein spots identified by mass spectrometry.

Bait	Accession No.	Protein name	pI[a]	MW[b]	Spot No.
Gpx3	P17695	Glutaredoxin 2	7.3	15.8	1
Gpx3	P54838	Dihydroxyacetone kinase1	5.1	62.2	7
Gpx3	P00358	Glyceraldehyde-3-phosphate dehydrogenase 2	6.9	35.8	9
Gpx3	P24813	AP-1-like transcription activator 1	7.0	45.7	10
Gpx3	P36100	Transcription initiation factor IIE subunit a1	5.9	101.9	11
$Gpx3^{C36S}$	P40029	Peptide methionine sulfoxide reductase	6.9	21.1	3
$Gpx3^{C36S}$	P43606	SSY protein 3	7.2	76.2	4
$Gpx3^{C82S}$	P38315	YAP1-binding protein 1	5.4	77.7	2
$Gpx3^{C82S}$	P17967	Protein disulfide isomerase 1	4.4	58.2	5
$Gpx3^{C82S}$	Q07471	Thiamine metabolism regulatory protein 3	6.0	68.3	8
Gpx3	P32288	Glutamine synthetase 1	5.9	41.7	6
$Gpx3^{C82S}$	P32288	Glutamine synthetase 1	5.9	41.7	6

[a,b]Theoretical value using ExPASY tool.

with yeast Gpx3, implying that our experiment worked well and is useful for mining the interacting protein of Gpx3. It is interesting that Gpx3 interacts with glutaredoxin 2 (Grx2), another antioxidant enzyme. Grxs are small proteins that are highly conserved and catalyze GSH-disulfide oxidoreductions [4]. Two genes encoding Grxs have been known in *S. cerevisiae*, Grx1 and Grx2, which share a high degree of similarity. Grx1 mutants are sensitive to oxidative stress caused by superoxide anion, whereas Grx2 mutants are vulnerable to stress induced by hydrogen peroxide. Furthermore, Grx2 accounts for most of the GSH-dependent oxidoreductase activity in yeast cells. From these results, we suggest that the interaction between Gpx3 and Grx2 may be involved in the fine regulation of redox status in yeast cells.

The formation of disulfide bonds involves the oxidation of thiols and the reduction and isomerization of nonproductive disulfides. These processes are catalyzed by protein disulfide isomerase (PDI: E.C. 5.3.4.1). PDI has two active-site domains, each with a Cys-Gly-His-Cys sequence motif that facilitates the transfer of disulfide bonds by forming a short-lived, covalent reaction intermediate with cysteines in substrate proteins [1, 16]. The *in vivo* reduction/oxidation (redox) state of the two active sites in PDI is critical for its biological function. From the possibility of interaction between Gpx3 and PDI, it is implicated that Gpx3 is involved in the redox control of active-site domains of PDI, thereby regulating the functions of PDI.

In conclusion, we identified several candidate Gpx3 interacting proteins by proteomic analysis. The list of candidate proteins provides an understanding of the novel function of Gpx3 as well as of detailed defense mechanisms against oxidative stress.

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