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Gpx1 is a stationary phase-specific thioredoxin peroxidase in fission yeast

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Abstract

The genome sequence of *Schizosaccharomyces pombe* reveals only one gene for a putative glutathione peroxidase (*gpx1*$^+$). The Gpx1 protein has a peroxidase activity but preferred thioredoxin to glutathione as an electron donor when examined *in vitro* and *in vivo*, and therefore is a thioredoxin peroxidase. Besides H_2O_2, it can reduce alkyl and phospholipid hydroperoxides. Expression of the *gpx1* gene was elevated at the stationary phase, and we found that it supported long-term survival of *S. pombe*. The mutant also exhibited some defect in the activity of aconitase, an oxidation-labile Fe–S enzyme in mitochondria. Activity of sulfite reductase, a labile Fe–S enzyme in the cytosol, was also dramatically lowered in the mutant in the stationary phase. The Gpx1 protein, without any obvious targeting sequence, was localized in mitochondria as well as in the cytosol. Therefore, Gpx1 must serve to ensure optimal mitochondrial function and cytosolic environment, especially in the stationary phase.

Keywords: Glutathione; Thioredoxin; Long-term survival; Oxidative stress

Sequence similarity divides the thiol-dependent peroxidases into two groups; glutathione peroxidase (Gpx)-type and peroxiredoxin/thioredoxin peroxidase (Prx/Tpx)-type proteins. The glutathione peroxidase (Gpx-1) was initially discovered as an antioxidative enzyme that reduces hydrogen peroxide by GSH in red blood cell [1]. It is the first enzyme discovered to contain selenocysteine coded by a stop codon TGA [2]. Most mammalian Gpxs contain selenium in the active site, whereas cysteine-replaced forms are found in plants, fungi, and bacteria as well as in mammals [3,4]. In addition to soluble hydroperoxides, some Gpxs (PHGpxs) have the ability to reduce phospholipids hydroperoxide, thus protecting membranes against lipid peroxidation [5,6]. Not only serving a protective role against oxidative stress, glutathione peroxidases have been implicated in various signal transduction and differentiation processes [3]. For example, Gpx3 in yeast *Saccharomyces cerevisiae* serves as a sensor for hydrogen peroxide, and the oxidized Gpx3 activates transcription factor Yap1, which regulates genes for response against hydrogen peroxide [7]. The substrate specificity of Gpx-type peroxidases is rather relaxed [8]. For example, Gpx2 and Gpx3 in *S. cerevisiae* both prefer thioredoxin to glutathione as reductants, and hence functionally are thioredoxin peroxidase even though their amino acid sequence is distant from the conventional Tpx or peroxiredoxins [7,9].

In fission yeast *Schizosaccharomyces pombe*, constant provision of reduced GSH is essentially required for the aerobic growth of the cell, primarily by protecting Fe–S clusters from oxidative damage in mitochondria [10]. Different from most other organisms which contain more than one isoforms of Gpx, the genome sequence of *S. pombe* predicts only one gene (*gpx1*$^+$) for a putative glutathione peroxidase (SpGpx1). Its expression is induced by hydrogen peroxide through the action of Atf1, a peroxide-responsive regulator in *S. pombe*, and thus confers resistance toward hydrogen peroxides [11]. However, no further details on its enzymatic activity and its role in the physiology of *S. pombe* have been reported. In this paper we characterize the enzyme activity of SpGpx1, and demonstrate

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that it is a thioredoxin-dependent peroxidase that resides both in the cytosol and mitochondria, and protects mitochondrial function such as respiration and oxidation-labile Fe–S enzyme activities. It also protects labile Fe–S enzyme in the cytosol and supports long-term survival of *S. pombe*.

Materials and methods

Strains and culture conditions. *Schizosaccharomyces pombe* strains ED665 (*h*$^-$ *ade6-M210 leu1-32 ura4-D18*) and ED668 (*h*$^+$ *ade6-M216 leu1-32 ura4-D18*) were used as parental strains to construct mutants. JY21d (*trx1::ura4*$^+$ in ED665), and JY31b (*trx2::ura4*$^+$ in ED668) [12] were used to generate *Δtrx1trx2* double mutant through mating and selection by tetrad analysis. Cells were routinely grown in yeast extract medium (YES) and synthetic minimal medium (EMM) as described by Alfa et al. [13].

Peroxidase activity assay. Peroxidase activity was determined as described previously with some modifications [7,14] by monitoring NADPH consumption through absorption at 340 nm. The assay buffer contained 50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.15 mM NADPH, and either a thioredoxin system (1.34 μM thioredoxin and 0.18 μM thioredoxin reductase from *Escherichia coli*), or a glutathione system (0.5–3 mM GSH and 2 μM glutathione reductase from *S. cerevisiae*). All the reagents and enzymes used for the assay were from Sigma Co. Phosphatidylcholine hydroperoxide (PHP) was generated by using soybean lipoxygenase and purified through C18 reverse-phase column (Sepak cartrige, Waters) by methanol elution as described previously [15]. Purified Gpx1 proteins (28 μg) (wild type or cysteine substitution mutant) were added to a 1 ml final volume and the reaction was started by adding 150 μM H_2O_2, 1.2 mM *t*-butyl hydroperoxide (*t*-BHP), or 80 μM phosphatidylcholine hydroperoxide (PHP).

Disruption of the gpx1$^+$ *gene*. We generated PCR fragment containing the entire coding region of the *gpx1*$^+$ gene using a mutagenic forward primer (CACATTGA CAAGCTTGTAACGTAA; HindIII site underlined) and a reverse primer (TGGCT GTTAAAGCTTCTGTATCTG; HindIII site underlined). The 2.80 kb HindIII-cut *gpx1*$^+$ coding region was cloned into pTZ18R vector, generating pTZ18R-GPX1. The *ura4*$^+$ gene of *S. pombe* [16] was inserted into the SnaBI/EcoRV site of the *gpx1*$^+$ gene in pTZ18R-GPX1, generating pTZ18R-GPX1::*ura4*$^+$. The HindIII fragment (3.93 kb) from pTZ18R-GPX1::*ura4*$^+$, which contains *ura4*$^+$ cassette (1.76 kb) flanked by *gpx1*$^+$ gene sequence, was used to transform a diploid cell (ED665 cross ED668). Following selection by the *ura4*$^+$ marker, the expected heterozygous (*gpx1*$^+$/*gpx1::ura4*$^+$) gene structure of the diploid transformant was confirmed by PCR and Southern hybridization. Through sporulation, a haploid mutant strain (ESG5; *h*$^-$ *ade6-M210 leu1-32 ura4-D18 gpx1::ura4*$^+$) was isolated.

Purification of recombinant Gpx1 proteins. The entire coding region of the wild type or the mutant *gpx1* gene cloned in pWH5 was amplified by PCR, cloned into pET15b, a hexa-histidine-tagging plasmid, and intro-

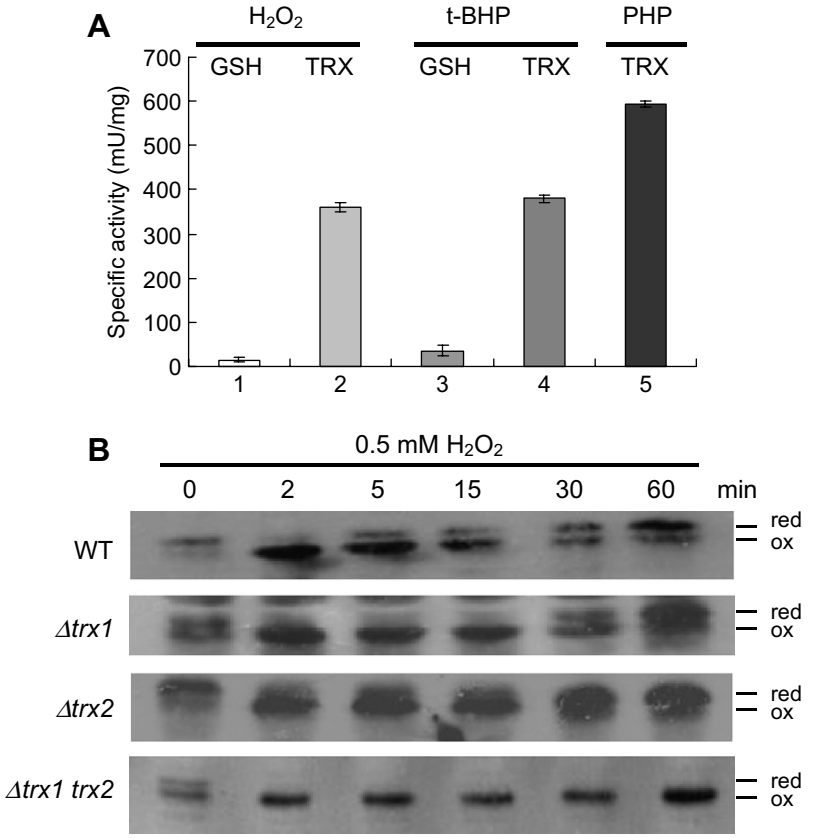

Fig. 1. Thioredoxin-dependent reduction of Gpx1 *in vitro* and *in vivo*. (A) Peroxidase activity of purified Gpx1 with various substrates. The peroxidase activity of the purified recombinant Gpx1 was measured using H_2O_2 (lanes 1 and 2), *t*-butylhydroperoxide (*t*-BHP; lanes 3 and 4), or phosphatidylcholine hydroperoxide (PHP; lane 5) as substrates. For electron donating system, NADPH and either a glutathione system (GSH and glutathione reductase; lanes 1 and 3) or a thioredoxin system (thioredoxin and thioredoxin reductase; lanes 2, 4, and 5) were used as described in Materials and methods. Average values with standard deviations from at least three independent experiments were presented. (B) Thioredoxin-dependent reduction of Gpx1 *in vivo*. The wild type and thioredoxin mutant cells (Δ*trx1*, Δ*trx2*, and Δ*trx1trx2*) grown to stationary phase in complex media (YES) were treated with 0.5 mM H_2O_2 for indicated lengths of time up to an hour, followed by treatment with 50 mM NEM. Total cell extracts were electrophoresed on non-reducing SDS–PAGE, and Gpx1 proteins were detected by Western blotting using anti-Gpx1 antibody raised from rabbit. The position of reduced (red) and oxidized (ox) forms was indicated.

duced into *E. coli* BL21-Gold (DE3). His-tagged Gpx1 proteins were prepared through standard protocol as recommended by the manufacturer.

Electrophoretic analysis of Gpx1 redox state in vivo. For *in vivo* analysis of Gpx1 redox state, stationary phase cells (wild type, *Δtrx1*, *Δtrx2*, and *Δtrx1trx2*) grown to OD_{595} of 8–10 were treated with 0.5 mM H_2O_2 for various lengths of time. They were then treated with 50 mM NEM for 5 min to alkylate free thiols before harvest. Cell extracts were electrophoresed on non-reducing or reducing 12% SDS–PAGE and analyzed by immunoblotting with polyclonal anti-Gpx1 antiserum raised from rabbits.

Subcellular fractionation. The cytosol and organelle fractions were prepared as described previously [17,10].

Fluorescence microscopy. The *gpx1*$^+$ gene was cloned into the pREP42EGFP-C plasmid [18] that links enhanced green fluorescence protein (EGFP) to the C-terminus of the cloned gene. The resulting plasmid (pREP42-Gpx1-EGFP) was introduced into a cell that contains red fluorescence protein (RFP)-tagged *sdh4*$^+$ gene encoding a succinate dehydrogenase subunit as a mitochondrial marker. Fluorescence images were captured by an LSM510 MLD confocal microscope (Carl-Zeiss). The green and red signals were detected at 488 and 553 nm, respectively, and merged by using the Zeiss LSM image browser.

Enzyme assays for aconitase, succinate dehydrogenase, and sulfite reductase. The wild type (JH43) and *Δgpx1* (ESG5) cells were grown in YES to stationary phases. Enzyme activities of aconitase and succinate dehydrogenase in organellar extracts and sulfite reductase in the cytosolic fraction were measured as described previously [19–21].

Results and discussion

Gpx1 reduces various hydroperoxides using the thioredoxin system as an electron donor

The Gpx1 protein from *S. pombe* was overproduced and purified from *E. coli*. Peroxidase activity was measured with various substrates. To determine its electron donor, we monitored activity in the presence of either glutathione (GSH) or thioredoxin (Trx) system. We found that Gpx1 reduced H_2O_2, *t*-butyl hydroperoxide (*t*-BHP), and a phospholipid (phosphatidylcholine) hydroperoxide (PHP), efficiently in the presence of Trx system consisting of thioredoxin (Trx) and Trx reductase (Fig. 1A). In contrast, the glutathione system consisting of GSH and GSH reductase did not provide significant peroxidase activity for Gpx1. Therefore, Gpx1 in *S. pombe* is not a glutathione peroxidase as was annotated, but is a thioredoxin peroxidase.

In order to evaluate the effect of thioredoxins *in vivo*, the redox state of Gpx1 was monitored in the wild type and mutant cells that lack either one or both of thioredoxin genes (*trx1, trx2*) present in *S. pombe*. Cells grown to early stationary phase were treated with 0.5 mM H_2O_2 for varying lengths of time, and analyzed for the redox status of Gpx1 through electrophoresis and Western blot. In the absence of oxidant, Gpx1 was detected mainly as a reduced form in the wild type (Fig. 1B). Oxidized form was detected at 2 min after exposure to H_2O_2, the majority of which returned to the reduced state after 30 min following H_2O_2 treatment. In the *Δtrx1* and *Δtrx2* single mutants that lack genes for either cytosolic (*trx1*) or mitochondrial (*trx2*) thioredoxin, oxidized Gpx1 returned to its reduced state at similar rates as the wild type. However, in the double mutant lacking both thioredoxins, more than half of Gpx1 existed as an oxidized form even in the absence of oxidant, and stayed oxidized for up to an hour following H_2O_2 treatment (Fig. 1B). These results clearly demonstrate that Gpx1 in *S. pombe* is reduced by the Trx system *in vivo*.

The gpx1$^+$ *functions primarily in the stationary phase*

We examined the expression of the *gpx1*$^+$ gene by Western blot analysis at exponential and stationary phases. Fig. 2A demonstrates that the level of Gpx1 protein is prominently elevated in the stationary phase. The increase in the protein level coincided with increase in mRNA as examined by Northern analysis (data not shown). We then examined whether Gpx1 contributes to the long-term survival of *S. pombe*. The wild type and *Δgpx1* cells were grown in YES for more than 10 days, and viable cells were counted by plating assay. We found that the *Δgpx1* mutant loses viability drastically after seven days, whereas the wild type sustains its viability with milder loss (Fig. 2B). Therefore, it is evident that Gpx1 contributes heavily to cell survival under stationary phase and long-term culture.

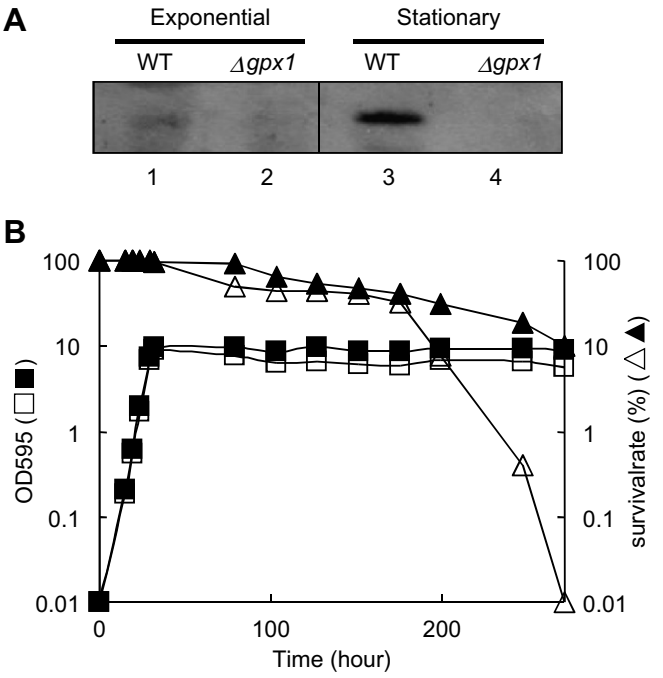

Fig. 2. Stationary phase-specific role of Gpx1. (A) Stationary phase expression of Gpx1. The wild type (JH43) and *Δgpx1* (ESG5) cells were grown in YES to exponential (lanes 1–2; OD595 ~3.2) and stationary (lanes 3–4; OD595 ~9) phases before harvest. Cell extracts containing 40 μg proteins were run on SDS–PAGE and immunoblotted with antibody against Gpx1 protein. (B) Contribution of Gpx1 in long-term survival of *S. pombe*. The wild type (filled symbols) and *Δgpx1* (opened symbols) cells were inoculated to OD_{595} of 0.01 in EMM media and then grown at 30 °C for more than 10 days. Both cell growth and viable cell numbers were monitored through measuring OD at 595 nm (squares) and colony counting on YES plates (triangles), respectively. Mean values from triplicate measurements were presented.

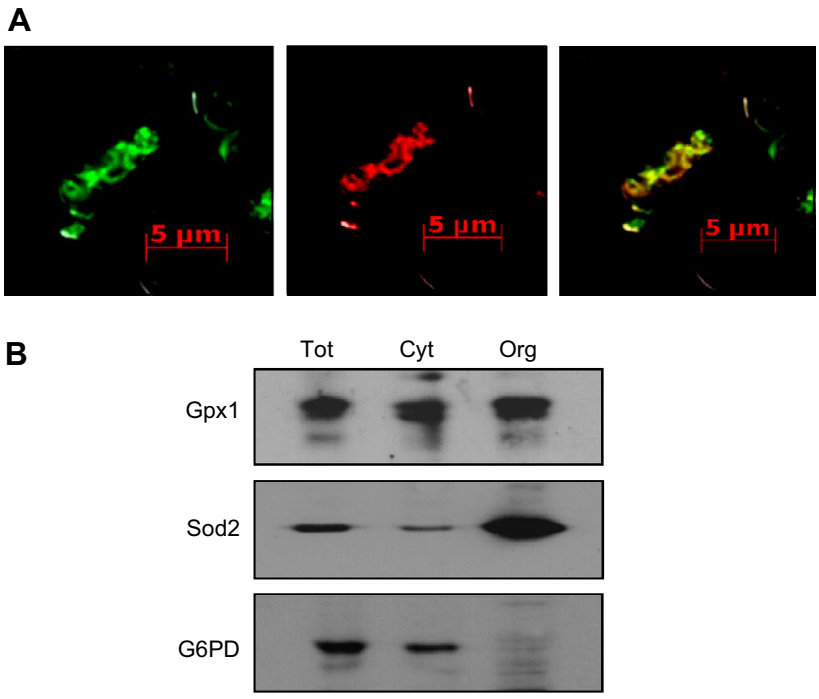

Fig. 3. Subcellular localization of Gpx1. (A) Fluorescence microscopy. Cells containing a choromosomally-integrated copy of the RFP-tagged *sdh4*$^+$ gene encoding a mitochondrial succinate dehydrogenase subunit were transformed with pREP42EGFP-C-based recombinant plasmid containing the GFP-tagged *gpx1*$^+$ gene. Fluorescent images were taken for cells grown in EMM to the stationary phase. (B) Subcellular fractionation. Cell extracts were prepared from the total (Tot), cytosolic (Cyt) and organellar (Org) fractions of wild type cells. In each fraction, Gpx1, mitochondrial MnSOD (Sod2) and cytosolic glucose-6-phosphate dehydrogenase (G6PD) proteins were detected by Western blotting.

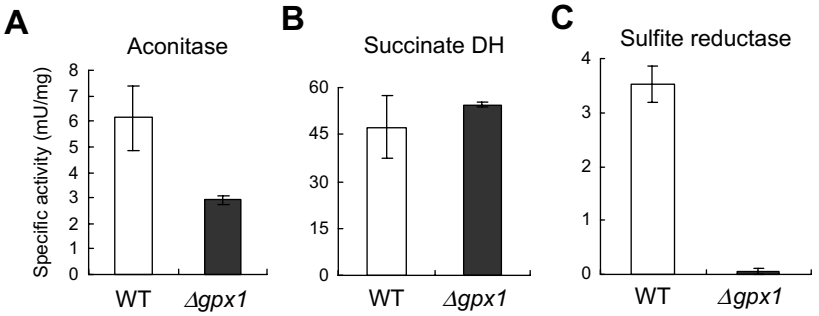

Fig. 4. Activity of iron–sulfur enzymes in *Δgpx1*. The enzyme activities of aconitase (A) succinate dehydrogenase (B) in the organelle fraction, and sulfite reductase (C) in the cytosolic fraction of cell extracts prepared from stationary-grown cells were measured as described in Materials and methods. Average values from three independent experiments were presented.

Gpx1 is located in mitochondria as well as in the cytosol

Typical prediction program such as PSORT-II (http://psort.nibb.ac.jp) predicts Gpx1 to be mainly localized in the cytosol (60.9% cytoplasmic, 21.7% nuclear, 4.3% cytoskeletal, 4.3% vacuolar, 4.3% mitochondrial, and 4.3% in vesicles of secretory system). Fluorescence microscopy of cells containing both the GFP-fused *gpx1*$^+$ and RFP-fused *sdh1*$^+$ encoding a mitochondrially located succinate dehydrogenase revealed that the green fluorescence image matched exactly with the red fluorescence from mitochondria (Fig. 3A). Some background (cytosolic) green fluorescence was also observed. We then examined distribution of Gpx1 protein in the cytosolic vs. organelle fractions through Western blotting. The proper separation of both fractions was confirmed by detecting mitochondrial MnSOD (Sod2) and cytosolic glucose-6-phosphate dehydrogenase (G6PD) [22]. Fig. 3B demonstrates that Gpx1 is present in both the organelle and cytosolic fractions. Therefore, the thioredoxin peroxidase encoded by the *gpx1*$^+$ gene is targeted to both mitochondria and the cytosol, and functions in both compartments.

Gpx1 contributes to maintain oxidant-labile iron–sulfur enzymes in mitochondria and cytosol

In addition to respiratory energy generation, mitochondria provide cells with Fe–S clusters for proteins in all com-

partments [23]. We therefore examined the effect of Gpx1 on various Fe–S enzymes. When mitochondrial Fe–S enzymes such as aconitase and succinate dehydrogenase were examined for their activities in the stationary phase, we found that the oxidant-labile aconitase was reduced in the mutant, whereas oxidant-insensitive succinate dehydrogenase was not (Fig. 4A and B). This implies that Gpx1 does not contribute to the biogenesis of Fe–S cluster, but to its maintenance, most likely through its anti-oxidative function. The activity of sulfite reductase, a cytosolic Fe–S enzyme sensitive to oxidants, was also greatly reduced in the mutant in the stationary phase (Fig. 4C). The sulfite reductase activity, however, was not affected in the exponential phase as expected (data not shown). We also observed that the level of oxidized proteins in the stationary phase, as monitored by antibody against carbonylated proteins, was significantly elevated in the mutant compared with the wild type (data not shown). These observations lead to the conclusion that Gpx1 functions as a key anti-oxidative surveillance system during stationary phase in *S. pombe*, protecting macromolecules such as lipids and oxidant-labile Fe–S proteins, using thioredoxins in both cytosolic and mitochondrial compartments (Trx1 and Trx2) as electron donors.

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