Saccharomyces cerevisiae Expresses Three
Phospholipid Hydroperoxide Glutathione Peroxidases

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Running Title: Phospholipid Hydroperoxide Glutathione Peroxidases in Yeast

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1The abbreviations used are: GPx, glutathione peroxidase; PHGPx, phospholipid hydroperoxide glutathione peroxidase; ROS, reactive oxygen species; t-BHP, tert-butyl hydroperoxide; PCOOH, phosphatidylcholine hydroperoxide; 18:3, linolenic acid; PUFA, polyunsaturated fatty acid; UI, fatty acid unsaturation index.
The GPX1, GPX2 and GPX3 genes of Saccharomyces cerevisiae have been reported previously to encode glutathione peroxidases (GPxs). We re-examined the sequence alignments of these proteins with GPxs from higher eukaryotes. Sequence identities, particularly with phospholipid hydroperoxide glutathione peroxidases (PHGPxs), were enhanced markedly by introduction to the yeast sequences of gaps that are characteristic of PHGPxs. PHGPx-like activity was detectable in extracts from wild type S. cerevisiae and was diminished in extracts from gpx1Δ, gpx2Δ and gpx3Δ deletion mutants; PHGPx activity was almost absent in a gpx1Δ/gpx2Δ/gpx3Δ triple mutant. Studies with cloned GPX1, GPX2 and GPX3 expressed heterologously in Escherichia coli confirmed that these genes encode proteins with PHGPx activity. An S. cerevisiae gpx1Δ/gpx2Δ/gpx3Δ mutant was defective for growth in medium supplemented with the oxidation-sensitive polyunsaturated fatty acid linolenate (18:3). This phenotype was more marked than sensitivity to H2O2. Unlike H2O2 toxicity, delayed toxicity of 18:3 towards gpx1Δ/gpx2Δ/gpx3Δ cells was correlated with the gradual incorporation of 18:3 into S. cerevisiae membrane lipids and was suppressible with α-tocopherol, an inhibitor of lipid peroxidation. The results show that the GPX genes of S. cerevisiae previously reported to encode GPxs encode PHGPxs, and that these enzymes protect yeast against phospholipid hydroperoxides as well as non-phospholipid peroxides. This is the first report of an organism that expresses PHGPx from more than one gene, and that produces PHGPx in the absence of a GPx.
Reactive oxygen species (ROS) are generated continually as by-products of aerobic metabolism in cells and their production may be enhanced by environmental insults, such as those posed by pro-oxidants, drugs and heavy metals. ROS are damaging to all cellular constituents including protein, DNA and lipid. Hence, cells possess a range of non-enzymatic and enzymatic defense systems to counter oxidative stress, including glutathione, thioredoxin, superoxide dismutase, and peroxidases such as catalase and glutathione peroxidase, which is commonly a selenoprotein (1). Peroxidases act by reducing hydroperoxides, e.g. H₂O₂. Many catalases do not react with larger hydroperoxides such as organic tert-butyl hydroperoxide (t-BHP), whereas glutathione peroxidases can act on both H₂O₂ and the larger hydroperoxides. Moreover, the principal antioxidant enzymes for H₂O₂ detoxification are generally considered to be the glutathione peroxidases rather than catalase, since catalase has a much lower affinity than glutathione peroxidase for H₂O₂ (2, 3).

Two principal forms of glutathione peroxidase (GPx) have been characterized in cells: classical GPx (cGPx, or GPx) and phospholipid hydroperoxide glutathione peroxidase (PHGPx) (other forms are plasma GPx and cytosolic gastrointestinal GPx) (1). PHGPx is monomeric and partly membrane associated, often evident in both soluble and membrane fractions, whereas GPx is tetrameric and soluble. Moreover, PHGPx is functionally distinct from GPx in that as well as the peroxides that are substrates of both enzymes, PHGPx reduces lipid hydroperoxides esterified to biomembranes. Phospholipid hydroperoxides are central intermediates in the lipid peroxidation chain reaction – lipid peroxidation being one of the major types of oxidative damage in cells, associated with membrane perturbation, inactivation of membrane proteins and cell lysis. Lipid peroxidation has also been linked to pathological conditions such as ischemic injury, atherosclerosis and carcinogenesis (4). Thus, PHGPx is the principal cellular enzyme
capable of membrane lipid peroxidation repair, and is generally considered the main line of enzymatic defense against oxidative membrane damage (5); GPx in cells may interfere with membrane lipid peroxidation only through concerted action with phospholipases (6, 7). There is also evidence that PHGPx reduces nucleic acid and protein hydroperoxides and is involved in structural and cell-signaling functions (8). Despite this, GPx appears to be more common than PHGPx, with no organism previously shown to possess more than one gene encoding PHGPx.

The experimental advantages of the yeast model *Saccharomyces cerevisiae* have been exploited extensively for advancing our understanding of cellular defenses against ROS (for reviews see 3, 9-11). GPx-like activity in yeast extracts has been known for some time (12, 13) and the completion of the yeast genome sequence in 1996 revealed three open reading frames predicted to encode GPx-like proteins. Inoue et al. (14) recently undertook the first characterization of these ORFs, which contain a cysteine residue at the conserved active-site position commonly occupied by selenocysteine in GPxs of higher organisms. One of the ORFs was induced by glucose starvation, and another by oxidative stress in a Yap1p-dependent manner. A triple deletion mutant lacking all three ORFs was hypersensitive to \( \text{H}_2\text{O}_2 \) and \( \text{t-BHP} \). Based principally on sequence analyses and observations of diminished activity against \( \text{t-BHP} \) in the deletion mutants, these workers proposed that all three ORFs encoded (selenium-independent) glutathione peroxidases. The genes were named *GPX1*, *GPX2* and *GPX3*.

Here, we re-examine the sequence alignments of the *GPX* products with other GPxs and show that the yeast proteins in fact exhibit greater similarity to PHGPxs than to GPxs. Furthermore, our biochemical and physiological studies confirm that *GPX1*, *GPX2* and *GPX3* encode PHGPxs and that these enzymes are required for cellular protection against lipid peroxidation. Thus, *S. cerevisiae* becomes the first organism known to express PHGPx from more than one gene, and the first shown to possess PHGPx in the absence of a GPx.
EXPERIMENTAL PROCEDURES

Yeast Strains and Preparation of Cell Extracts — Saccharomyces cerevisiae BY4741

(MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and derivative single deletion-mutants gpx1Δ, gpx2Δ,
gpx3Δ, ccp1Δ, ctt1Δ, tsalΔ, sod1Δ and sod2Δ were obtained from Euroscarf (Frankfurt,
Germany). The gpx1Δ/gpx2Δ/gpx3Δ triple mutant was constructed by short flanking homology
PCR (15) using the URA3, His3MX6 and kanMX6 markers for gene disruption. For preparation
of cell extracts, organisms were cultured to mid-/late-exponential phase (OD600 ~1.0) in liquid
YEPD medium, as described previously (16), and collected by centrifugation followed by two
washes with 0.85% (w/v) NaCl. Cells were resuspended in lysis buffer (100 mM Tris-HCl pH
8.0, 1 mM PMSF, 0.03 mM leupeptin) and were disrupted with glass beads (0.5-mm diameter) by
using a mini-bead-beater (Biospec Products). Cell debris was removed by centrifugation (12,000
x g, 10 min, 4°C) and supernatants retained for enzyme assays. Protein concentrations in the
supernatants were determined using the Bradford assay (17).

Preparation and Purification of Recombinant Protein — The full-length ORFs of GPXI
(YKL026c), GPX2 (YBR244w) and GPX3 (YIR037c) were tagged with BamHI and XhoI sites
(underlined below) during amplification by PCR, using S. cerevisiae genomic DNA as template.
The primers used were: GPXI, 5’-CCGCTCGAGATGCAAGAATTTTATTC-3’ and 5’-
CGCGGATCCCTTAATCTGTTCTTCTGG-3’; GPX2, 5’-
CCGCTCGAGATGACCACATCTTTT-3’ and 5’-CGCGGATCCCATTATTACTTACAGG-3’;
GPX3, 5’-CCGCTCGAGATGTCAAGAATTTCATCT-3’ and 5’-
CGCGGATCCCTATTCCACCTCTTTC-3’. The amplified fragments were inserted between the
BamHI and XhoI sites of the plasmid vector pET14b (Novagen), to give in-frame fusion with a 6-
His tag. Sequence fidelity of all products was routinely confirmed by automated DNA sequence analysis. For experiments, recombinant plasmids were transformed into *E. coli* BL21(DE3), and ampicillin-resistant colonies cultured in LB<sub>amp</sub> broth to OD<sub>600</sub> ∼0.6 before induction of recombinant proteins by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. Two hours after induction, cells were harvested by centrifugation (6,000 x g, 5 min, 4°C), washed twice in 0.85% NaCl, and resuspended in lysis buffer. Cells were disrupted by three freeze-thaw cycles followed by sonication, and cell debris sedimented by centrifugation (12,000 x g, 10 min, 4°C). Protein concentrations in the supernatants were determined using the Bradford assay (17). Extracts were analyzed for the soluble recombinant protein by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using standard protocols (18).

To solubilize the *GPX1* gene product, cell disruption was performed in lysis buffer supplemented with TritonX-100 [0.12 % (v/v)]. After centrifugation of extracts as described above, the supernatant was retained and the pellet resuspended in lysis buffer supplemented with tergitol (Nonidet P-40; Sigma) at a final concentration of 0.5% (v/v) and then sonicated briefly.

The His-tagged protein products of *GPX2* and *GPX3* were purified from cell extracts according to manufacturer’s instructions using 1 mL HiTrap chelating affinity columns (Amersham Pharmacia Biotech), charged with 0.1 M NiSO₄. A separate column was used for each recombinant protein. The columns were equilibrated with 0.02 M sodium phosphate, 0.5 M NaCl pH 7.4, and washed with 0.02 M sodium phosphate, 0.5 M NaCl, 0.05 M imidazole at pH 7.4. The purified proteins were eluted into ∼0.5 mL fractions with 0.02 M sodium phosphate, 0.5 M NaCl, 0.5 M imidazole at pH 7.4. Fractions containing the protein peaks (17) were assayed immediately for enzymatic activity.
**Enzyme Assays** — PHGPx reduces phospholipid hydroperoxides to their corresponding hydroxyphospholipids, with simultaneous oxidation of NADPH that can be monitored *in vitro* with spectrophotometry (19, 20) (electrons are transferred from NADPH to glutathione by glutathione reductase, which is included in assays). The phosphatidylcholine hydroperoxide (PCOOH) substrate was generated by the hydroperoxidation of phosphatidylcholine (Type III/S; Sigma) dispersions in deoxycholate, using soybean lipoxygenase, exactly as described by Ursini et al. (19) with the modifications of Weitzel et al. (20). PCOOH was purified by passage through Oasis HLB 3cc cartridges (Water Associates, Milford, MA) with methanol elution (21).

Enzymatic activities in crude cell extracts or of purified recombinant protein were assayed in reaction buffer comprising 100 mM Tris-HCl (pH 7.4), 1 mM NaN₃, 5 mM EDTA, 0.3 mM NADPH, 3 mM GSH, 0.12% Triton X-100 and 2 units/ml GSH reductase. Protein was added to 1 ml reaction final-volumes and after 3 min equilibration with continuous stirring the reaction was started by the addition of PCOOH or t-BHP substrate, according to standard protocols (20, 21). NADPH oxidation was recorded spectrophotometrically as a linear change in absorbance at 340 nm, between 3 and 5 min incubation after the start of reactions. Rates of NADPH consumption were corrected for: (a) GSH-independent NADPH-oxidizing activity, by omitting glutathione; b) peroxide-independent NADPH-oxidizing activity, by omitting substrate; c) non-enzymatic NADPH oxidizing activity, by omitting protein. One unit of activity was defined as the amount of protein (extract) required to oxidize 1 µmol of NADPH per min at 25°C. Where specified, the phospholipase inhibitors indomethacin and neomycin sulfate were added 5 min prior to the addition of substrate, to final concentrations of 200 and 100 µM respectively.

**Linolenate and \(H₂O₂\) Toxicity** — Organisms were cultured to mid-/late-exponential phase (OD₆₀₀ ~2.0) in liquid YEPD medium. For spotting experiments, the cultures were adjusted to
OD<sub>600</sub>'s of ~2.5, 0.25, 0.025, 0.0025 and 0.00025 for each strain. Samples (4 µl) from each dilution were spotted on YEPD agar, supplemented with filter-sterilized linolenic acid (18:3) or H<sub>2</sub>O<sub>2</sub> as specified. For all experiments involving 18:3, medium was prepared with tergitol to solubilize fatty acids; the final tergitol concentration was 1% (wt/vol). Control media and H<sub>2</sub>O<sub>2</sub>-supplemented media were also supplemented with tergitol, which has no adverse effect on yeast growth (16). For experiments in YEPD liquid medium (supplemented as above), cells were suspended to OD<sub>600</sub> ~0.2 and incubated with shaking (120 rev min<sup>-1</sup>), as described previously (16). Where specified, cultures were supplemented with α-tocopherol (200 µM) 30 min prior exposure of cells to 18:3 or H<sub>2</sub>O<sub>2</sub>. Colony forming units (CFUs) were determined after appropriate dilution and incubation on YEPD agar for 4 d at 30ºC.
RESULTS

The Peptide Sequences Encoded by GPX1, GPX2 and GPX3 Exhibit PHGPx-like Features

— Inoue et al. (14) based their initial identification of the open reading frames YKL026c, YBR244w and YIR037w as glutathione peroxidases, on sequence alignment data. Analysis of these alignments reveals a mean identity of the three yeast sequences with human GPx (GPx1) of ~23%. However, the homology from each of those alignments deteriorated after amino acid residue 78, with a mean identity of only ~7% with human GPx over the ~85 C-terminal residues (14). When we re-examined the sequence alignments using ClustalW software, two gaps were introduced to the \textit{S. cerevisiae} sequences commencing at residues 85 and 116 (Fig. 1). This realignment increased the mean identity between residues 78 and the C termini with the corresponding portion of human GPx from ~7% to ~27%, giving an increase in the mean overall identity with human GPx from ~23% to ~33%. Moreover, this revision restored the alignment of the third member of the GPx catalytic triad, tryptophan (W-125) (22, 23), with that of other GPxs (Fig. 1), while also increasing the homology in regions corresponding to secondary structures such as \( \alpha \)-helices and \( \beta \)-sheets (5, 22). The gaps introduced to the yeast sequences occur at the same positions as gaps that are conserved among PHGPx enzymes when aligned with GPxs (Fig. 1), and that are considered to distinguish this subfamily from GPxs (24). Consistent with this, there was also greater sequence homology of the yeast proteins with PHGPx than with GPx of other organisms. The products of \textit{S. cerevisiae GPX1, GPX2 and GPX3} exhibited 36%, 41% and 40% identity, respectively, with human PHGPx, and a mean ~47% overall identity with \textit{Arabidopsis thaliana} PHGPx (Fig. 1). Thus, our sequence alignments indicated that the protein products of \textit{GPX1, GPX2 and GPX3} in \textit{S. cerevisiae} were more likely to be PHGPxs than GPxs.
The Protein Products of GPX1, GPX2 and GPX3 Exhibit PHGPx Activity — To test whether GPX1, GPX2 and GPX3 encoded active PHGPx-like enzymes, we measured enzymatic activities in extracts from yeast deletion strains defective for each gene as well as for all three genes (Fig. 2). Only functionally active PHGPx-like enzymes should display activity against both the t-BHP and PCOOH substrates. In control reactions, we routinely confirmed that commercially purified GPx (from bovine erythrocytes; Sigma) exhibited strong activity with t-BHP but no detectable activity with PCOOH. In agreement with the previous work (14), we observed marked reductions in activity against the GPx substrate (t-BHP) in extracts from both the gpx3Δ and the triple knockout strains compared to the wild type; GPX1 and GPX2 deletion had lesser effect. We also detected PHGPx-like activity against the PCOOH substrate in the wild type strain (Fig. 2). Moreover, this PHGPx-like activity was diminished markedly in the gpx2Δ (~45 % reduction), gpx3Δ (~58 % reduction) and the triple knockout (~81 % reduction) mutants, indicating that the proteins encoded by the deleted genes were likely to be PHGPxs.

To characterize the products of GPX1, GPX2 and GPX3 further, we expressed recombinant forms of the three proteins heterologously using an E. coli overexpression system. E. coli lacks GPx and PHGPx-like activities (25), and the negligible PHGPx activity that we detected in crude lysates from control cells transformed with empty vector was consistent with this. Cells overexpressing GPX1, GPX2 and GPX3 exhibited PHGPx-like activities that were approximately 4-fold, 8-fold and 11-fold higher than background, respectively (Fig. 3A). This sequence of increasing activity, with the GPX3 product apparently the most active, was consistent with our observations with the yeast mutants (Fig. 2). We also verified activity against t-BHP in the E. coli lysates (data not shown), confirming that the yeast protein products were active also against non-phospholipid hydroperoxides.
We purified the recombinant GPX products so that we could rule out the possibility that they might be acting in conjunction with cellular phospholipases to give the effect of PHGPx-like activity in crude E. coli extracts (6, 7). The recombinant protein products of GPX2 and GPX3 were expressed by E. coli in soluble form, facilitating purification by affinity chromatography. Based on PHGPx activity, the products of GPX2 and GPX3 were purified 2.1 and 2.8-fold, respectively, relative to crude cell lysates (Fig. 3A). Protein bands corresponding to the recombinant products were evident from SDS-PAGE at ~23 and ~21 kDa, respectively (Fig. 3B). The GPX1 product was produced by E. coli in an insoluble form. We succeeded in solubilizing this protein using tergitol (see Experimental Procedures), which allowed detection of PHGPx-like activity in whole cell extracts (above), but we were unable to produce purified protein.

Since the product of GPX2 was not purified to full homogeneity, we used indomethacin and neomycin sulfate (26, 27) to inhibit any residual phospholipase activity. This also served as a control against bifunctional glutathione peroxidase / phospholipase A2 enzyme, such as has been described in mammalian cells (7). NADPH reducing activity in the PHGPx assays was not affected by the phospholipase inhibitors (Fig. 3B). Thus, phospholipase activity does not contribute to the PHGPx-like activity exhibited by these proteins.

Our sequence analyses and biochemical data collectively show that the S. cerevisiae ORFs YKL026c (GPX1), YBR244w (GPX2) and YIR037w (GPX3) have broader substrate specificities than originally thought and should be termed phospholipid hydroperoxide glutathione peroxidases. For the remainder of this paper, we will refer to these proteins as PHGPx1p, PHGPx2p and PHGPx3p, respectively.

**PHGPx is Required for Growth in the Presence of Linolenate** — It was shown previously that the yeast GPX products confer protection against H$_2$O$_2$ (14), as is the case for many
peroxidases. The ability to reduce phospholipid hydroperoxides, and impede the progress of lipid peroxidation after initiation, is the principal function that distinguishes PHGPx from GPx. To see whether we could detect such function at the phenotypic level, we sought to enrich wild type and \textit{gpx}\textsuperscript{$\Delta$} \textit{S. cerevisiae} with the oxidation-sensitive polyunsaturated fatty acid (PUFA) linolenate (18:3). \textit{S. cerevisiae} does not synthesize PUFAs naturally, so its membrane phospholipids normally contain only saturated and monounsaturated fatty acids. However, 18:3 can comprise >60\% of total membrane fatty acids in \textit{S. cerevisiae} cultured in the presence of 1 mM 18:3, and this normally has no deleterious effect on growth (16, 28). Moreover, since 18:3-enriched cells are sensitized to stress-induced lipid peroxidation, comparison of these cells with non-enriched cells provides an excellent means of testing whether lipid peroxidation is involved in a stress/toxicity mechanism (16, 28, 29). In this work, while attempting to generate 18:3-enriched organisms, we found that the growth of the \textit{gpx1}\textsuperscript{$\Delta$}/\textit{gpx2}\textsuperscript{$\Delta$}/\textit{gpx3}\textsuperscript{$\Delta$} mutant was almost fully abolished in the presence of 1 mM 18:3 and was severely impaired at 0.1 mM 18:3 (Fig. 4). The \textit{gpx} single mutants did not exhibit diminished colony formation in the presence of 18:3, although colonies of the \textit{gpx3}\textsuperscript{$\Delta$} strain were smaller at 1 mM 18:3. As reported previously (14), the \textit{gpx1}\textsuperscript{$\Delta$}/\textit{gpx2}\textsuperscript{$\Delta$}/\textit{gpx3}\textsuperscript{$\Delta$} mutant was sensitive to H\textsubscript{2}O\textsubscript{2}, but the degree of sensitization to 18:3 was greater: 1 mM 18:3 and 1 mM H\textsubscript{2}O\textsubscript{2} had similar inhibitory effects on \textit{gpx1}\textsuperscript{$\Delta$}/\textit{gpx2}\textsuperscript{$\Delta$}/\textit{gpx3}\textsuperscript{$\Delta$} cells, but only the latter treatment affected the growth of wild type cells also (Fig. 4).

To substantiate that protection against 18:3 was specific to PHGPx and is not a feature shared by non-phospholipid targeting peroxidases, we tested \textit{S. cerevisiae} mutants carrying deletions in the \textit{CCP1} (cytochrome c peroxidase), \textit{CTT1} (cytosolic catalase) and \textit{TSA1} (thioredoxin peroxidase) genes. The growth of these mutants was not affected by the presence of
18:3 in the growth medium (data not shown). We also confirmed the 18:3-insensitivity of sod1Δ and sod2Δ cells, as reported previously (29).

While monitoring *S. cerevisiae gpx1Δ/gpx2Δ/gpx3Δ* in 18:3-supplemented liquid medium, we noted that differences in OD_{600} between wild type and mutant cultures only became apparent after ~4 h incubation. Since OD measurements do not report directly on viability, to examine further this delayed inhibitory effect we monitored colony forming ability at intervals during growth in 18:3-supplemented liquid medium (Fig. 5A). Reductions in colony forming ability became apparent after 2 h at 2 mM 18:3 and only after 6 h at 1 mM 18:3, with a diminished rate of growth evident after 3 h in the latter case. This delay in 18:3 toxicity was in keeping with the slow incorporation of exogenous 18:3 into *S. cerevisiae* membrane lipids which takes place principally over the initial 8 h of incubation at 1 mM 18:3, as reflected by fatty acid unsaturation index values (Fig. 5A, inset). In contrast to 18:3, H_{2}O_{2} toxicity towards *S. cerevisiae gpx1Δ/gpx2Δ/gpx3Δ* was evident within 20 min and, at 0.5 mM H_{2}O_{2}, the viability of the mutant diminished to its lowest level after 1 h incubation (Fig. 5B). The results show that 18:3 toxicity occurred after 18:3 had been incorporated into membrane lipids in appreciable amounts, consistent with protection by PHGPx against 18:3 hydroperoxides formed in membranes rather than against free hydroperoxides as in the case of H_{2}O_{2}.

To corroborate that the 18:3-sensitivity of the *S. cerevisiae gpx1Δ/gpx2Δ/gpx3Δ* mutant was related to defective suppression of lipid peroxidation in 18:3-rich membranes, we treated cells with α-tocopherol (vitamin E). α-Tocopherol is a highly hydrophobic antioxidant that acts almost exclusively against oxy-radicals generated in biological membranes, where it breaks the lipid peroxidation chain reaction (4, 30). α-Tocopherol conferred partial protection against 18:3 toxicity in *S. cerevisiae gpx1Δ/gpx2Δ/gpx3Δ*. Thus, the onset of discernible 18:3-dependent
growth inhibition was delayed from approximately 3.5 h to 5 h in α-tocopherol-treated cells (Fig. 6A). Furthermore, the cell yield after 10 h with 18:3 was increased >2-fold in the presence of α-tocopherol, consistent with lipid peroxidation being a principal mechanism of 18:3 toxicity. α-Tocopherol treatment had no effect on H2O2-dependent inhibition of growth of the mutant cells (Fig. 6B), confirming that the action of α-tocopherol against 18:3 toxicity does not reflect a generalized antioxidant action.
DISCUSSION

A relationship between cellular glutathione and the resistance of yeast to certain products of lipid peroxidation is known (31, 32). This study reveals new functions that may establish part of this relationship. Marked activity against phospholipid hydroperoxides is unique to PHGPx among cellular enzymes. Thus, unlike GPx, PHGPx is capable of directly reversing the process of membrane lipid peroxidation. We have shown that the three *S. cerevisiae* genes formerly proposed to encode GPxs (*GPX1, GPX2* and *GPX3*) in fact encode PHGPxs. This conclusion is based on several lines of evidence. First, the yeast proteins show greater homology with other PHGPxs than GPxs. Second, when aligned to other GPx sequences the yeast proteins exhibit the sequence gaps that are considered to distinguish PHGPx from GPx. These gaps correspond precisely to the subunit interaction sites of GPx, and so may account for the monomeric structures of PHGPxs (24). The markedly improved homology with other GPx sequences that the gaps introduce was not noted previously (14), and this presumably relates to the alignment software used by those authors. This led to a misalignment from around amino acid 78 in their study and a subsequent breakdown in homology. The apparent molecular weights of the yeast proteins were consistent with those of mammalian homologs (19), but greater than the predicted weights (14) as also seen in the enzymes of other organisms (33), which lends further support to posttranslational modification of PHGPx (24). Third, and crucially, the yeast enzymes were active against a phospholipid hydroperoxide substrate, with apparent relative activities increasing in the order PHGPx1p < PHGPx2p < PHGPx3p. Fourth, *gpxΔ S. cerevisiae* were 18:3 sensitive.

Toxicity that arises from culturing of *S. cerevisiae* with PUFAs such as 18:3 is generally accepted to result from enhanced susceptibility of PUFA-rich membranes to lipid peroxidation.
The potential fragility of PUFA-rich \textit{S. cerevisiae} may be revealed by incubation under conditions that promote oxidative stress (28, 34) or in mutants that are defective in protection against lipid peroxidation (29). Thus, the 18:3 sensitivity of \textit{S. cerevisiae coq3}∆ mutants previously implicated reduced coenzyme Q as a lipid soluble antioxidant that confers specific protection against lipid peroxidation in yeast (29). The marked sensitivity to 18:3 of PHGPx-deficient \textit{S. cerevisiae}, but not of \textit{S. cerevisiae} mutants defective for several other antioxidant functions (e.g. other peroxidases), was consistent with the phospholipid hydroperoxide-reducing activity of PHGPx conferring specific protection against 18:3-associated membrane-lipid peroxidation. The delayed toxicity of 18:3 in \textit{gpx1}∆/\textit{gpx2}∆/\textit{gpx3}∆ cells – similar to that seen in \textit{coq3}∆ \textit{S. cerevisiae} (29) – as well as the suppression of toxicity with \(\alpha\)-tocopherol, further implicate membrane-lipid peroxidation as the primary mechanism of 18:3 toxicity in PHGPx-deficient cells. Although only partial in both studies, the suppression of 18:3 toxicity by \(\alpha\)-tocopherol was greater in \textit{coq3}∆ cells (29) than in \textit{gpx1}∆/\textit{gpx2}∆/\textit{gpx3}∆ cells. This is in keeping with the synergistic action of PHGPx and \(\alpha\)-tocopherol in preventing lipid peroxidation (21). In contrast to 18:3, protection against \textit{H}_2\textit{O}_2 – which diffuses rapidly in cells and across biological membranes (4), hence exerting toxicity more rapidly than 18:3 – is common to many peroxidases. Since \(\alpha\)-tocopherol did not affect the \textit{H}_2\textit{O}_2 sensitivity of \textit{gpx1}∆/\textit{gpx2}∆/\textit{gpx3}∆ cells, lipid peroxidation seems unlikely as a principal cause of \textit{H}_2\textit{O}_2 toxicity. This also corroborates that PHGPx-dependent direct reduction of \textit{H}_2\textit{O}_2 is more likely than phospholipid-targeted peroxidase action as the PHGPx activity mediating protection against \textit{H}_2\textit{O}_2. Activity against oxidative damage generated within lipid membranes as well as against soluble inorganic peroxides is fully in keeping with the generally accepted biochemical activities of PHGPx enzymes (19). Our study also shows that the lower activity generally seen in PHGPxs with a
cysteine residue rather than selenocysteine at the active site (25) clearly does not preclude
significant physiological function.

Some functional redundancy between the products of GPX1, GPX2 and GPX3 was evident
from the >1000-fold reduction in colony forming ability of the gpx1Δ/gpx2Δ/gpx3Δ mutant at 1
mM 18:3, compared with no decline in the numbers of colonies formed by the respective single
mutants under the same conditions. Thus, the sensitivity phenotype is not additive, and even the
low PHGPx activity evident in the gpx3Δ single mutant (GPX3 deletion was associated with the
largest single reduction in cellular PHGPx activity) was sufficient to confer almost full resistance
to 18:3. Such overlapping function raises the question why S. cerevisiae should maintain and
express three GPX genes, with associated energetic costs, when one or two enzymes might be
sufficient. The answer probably lies at least partly in the differential regulation of these genes.

GPX1 is induced by glucose starvation, whereas GPX2 is induced by oxidative stress in a Yap1p-
dependent manner (14). Expression of GPX3 was not induced by any of the stresses tested by
these workers and this evidence, combined with the greater constitutive activity of PHGPx3p
[this paper, and (14)], suggests that basal expression of PHGPx3p could underpin a constitutive
antioxidant function during normal metabolism in S. cerevisiae (14).

That the promoter regions of the genes encoding the PHGPxs respond to discrete stimuli is
indicative of the likely importance of PHGPx in S. cerevisiae. The expression of three PHGPxs,
combined with the absence of a conventional GPx, is to our knowledge unique in S. cerevisiae.
Moreover, it suggests that S. cerevisiae has been under a particular selective pressure to evolve
and sustain a high capacity for enzymatic repair of membrane lipid peroxidation, specifically, in
response to various stresses; the much greater sequence identity between the S. cerevisiae
PHGPxs than with PHGPxs from other organisms [(see Fig. 1 and (14)] is in keeping with gene
duplications that arose after the evolutionary divergence of *S. cerevisiae* from its common ancestor with higher eukaryotes. Paradoxically, *S. cerevisiae* is unusual among eukaryotes in that, owing to the absence of the required desaturases, its membrane phospholipids are normally devoid of PUFAs (16). Thus, one might expect that *S. cerevisiae* should be less susceptible than many other organisms to potential damage from lipid peroxidation. Nonetheless, lipid peroxidation does occur in *S. cerevisiae* (28, 29, 35) and has been shown to account for membrane damage and killing, for example during metal-induced oxidative stress (28). Therefore, one possible explanation for the absence of PUFA synthesis together with the expression of three PHGPx enzymes could be that *S. cerevisiae* is prone to particularly high ROS fluxes generated in or directed towards membranes than other organisms. In such a model, both PUFA-lacking membranes and high PHGPx activity (possibly along with the capacity to ferment) might be considered evolutionary adaptations to help *S. cerevisiae* combat such fluxes.

While much work with *S. cerevisiae* is readily justified by this organism’s similarities with higher systems, this study reveals a new feature that (currently) is unique to *S. cerevisiae*. The further study of the three PHGPxs of *S. cerevisiae* may help to address important questions about the selective pressures that dictated their evolution over other antioxidant systems such as GPxs and, moreover, the cellular requirement for PHGPx in protection against lipid peroxidation specifically. This report reveals PHGPx-dependent reduction of phospholipid hydroperoxides as a new and potentially important component of the repertoire of defenses available to *S. cerevisiae* in combating oxidative stress. It also seems likely that PHGPx activity is important for the health of higher organisms in which PUFAs occur as natural components of membrane lipids.

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   *Prostaglandins* **47**, 55-59


FIG. 1. Alignment of the amino acid sequences of GPx/PHGPx from *S. cerevisiae* and other eukaryotic sources. The amino acid sequences of the C-terminal halves (aa 77 – 167 for the *GPXI* product) of the *S. cerevisiae* *GPX1*, *GPX2* and *GPX3* products were aligned with the corresponding regions of homologs using MacVector v.6.01 ClustalW alignment software [the alignments from aa 1 – 76 were the same as presented by Inoue et al. (14)]. GenBank™ accession numbers of the sequences shown are: yeast (PH)GPx1, NP 012899; (PH)GPx2, NP00983; (PH)GPx3, NP012303; human GPx, P07203; rat GPx, P04041; human PHGPx, AAC32261; rat PHGPx, JC4332; *Arabidopsis* PHGPx, T04207. Residues identically conserved in at least one of the yeast sequences and in at least one of the sequences from other organisms are boxed in black. The third member of the GPx catalytic triad (tryptophan, W-125) is indicated by an asterisk.

FIG. 2. PHGPx activities in *S. cerevisiae gpxΔ* mutants. Enzyme activities were assayed in extracts from the various strains as described in the Experimental Procedures section, using the PHGPx-specific substrate phosphatidylcholine hydroperoxide (PCOOH) (□) or the general GPx substrate tert-butyl hydroperoxide (t-BHP) (■). Activities were normalized to percentages of activities obtained with extracts from wild type cells, which were in the range 30 – 50 mU/mg protein. The data shown are means from three independent experiments ± SD.
FIG. 3. **Over-expression of GPX1, GPX2 and GPX3 in E. coli.**  

A, PHGPx activities in extracts from *E. coli* DE3 after induction of heterologous *GPX1, GPX2* or *GPX3* from *S. cerevisiae*.  

DE3, *E. coli* transformed with empty vector.  Activities were determined with crude *E. coli* extracts (□), purified enzyme (■), purified enzyme + indomethacin (□□□) or + neomycin sulfate (□□□).  Values are means from three independent experiments ± SD.  

B, Separation by SDS-PAGE of protein extracts from *E. coli* overexpressing *GPX2* or *GPX3* (lanes 1 and 3), and after purification of the gene products as described in the Experimental Procedures (lanes 2 and 4).  PHGPx1p was expressed by *E. coli* in insoluble form.

FIG. 4. **Sensitivity of GPX-deficient S. cerevisiae mutants to 18:3 and H2O2.**  

Dilutions of decreasing cell concentration (from OD$_{600}$ ~ 2.5 to ~0.00025) were inoculated in 4 µl spots from left to right on each plate for each strain.  Typical results from one of three independent experiments are shown.

FIG. 5. **Time courses of 18:3 and H2O2 toxicities towards S. cerevisiae**

*gpx1Δ/gpx2Δ/gpx3Δ*.  Exponential-phase cells were inoculated in YEPD medium supplemented with 0 mM (○), 1 mM (●) and 2 mM (□) 18:3 (*A*), or 0 mM (○), 0.5 mM (●) and 1 mM (□) H$_2$O$_2$ (*B*).  Viability was monitored at intervals as colony forming ability on YEPD agar.  Mean values from three replicate samples are shown, ± SDs where these exceed the dimensions of the symbols.  Typical results from one of three independent experiments are presented.  The inset is adapted from (28) and shows changes in the fatty acid unsaturation index (UI, mean number of double bonds per fatty acid) during culturing of *S. cerevisiae* with 1 mM 18:3.
FIG. 6. Effect of α-tocopherol on the sensitivity of *S. cerevisiae* gpx1Δ/gpx2Δ/gpx3Δ to 18:3 and H2O2. Cells were grown in YEPD medium in the absence (open symbols) or presence (closed symbols) of 200 μM α-tocopherol. The medium was supplemented with 0 mM (○,●) and 1 mM 18:3 (□,■) (A), or 0 mM (○,●) and 0.5 mM H2O2 (□,■) (B). Mean OD600s from three replicate incubations are shown; SDs were smaller than the dimensions of the symbols. Typical results from one of three independent experiments are presented.
<table>
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<th>Protein Type</th>
<th>Sequence</th>
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<td>Arabidopsis PHGPx</td>
<td>--------LFGDKIKNWEAKFLVDKGNV8RFAPTRSPLSIEKDVKLLGVTAK</td>
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</table>
FIGURE 4

Wild type

Wild type

1.0 mM H₂O₂
FIGURE 6

![Graphs showing log_{10} (OD_{600}) vs. Time (h) for two conditions labeled A and B.](image-url)
Saccharomyces cerevisiae expresses three phospholipid hydroperoxide glutathione peroxidases
Angela M. Avery and Simon V. Avery

J. Biol. Chem. published online July 9, 2001

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