Genetic dissection of the phospholipid hydroperoxidase activity of yeast Gpx3 reveals its functional importance

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Running Title: Phospholipid hydroperoxidase function of yeast Gpx3

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1The abbreviations used are: GPx, glutathione peroxidase; PHGPx, phospholipid hydroperoxide glutathione peroxidase; ROS, reactive oxygen species; GSH, reduced glutathione; t-BHP, tert-butyl hydroperoxide; PCOOH, phosphatidylcholine hydroperoxide; 18:3, linolenic acid; PUFA, polyunsaturated fatty acid; CRR, cyanide resistant respiration
Saccharomyces cerevisiae expresses multiple phospholipid hydroperoxidase (PHGPx)-like proteins in the absence of a classical glutathione peroxidase (cGPx), providing a unique system for dissecting the roles of these enzymes in vivo. The Gpx3 (Orp1/PHGpx3) protein transduces the hydroperoxide signal to the transcription factor Yap1, a function that could account for most GPX-dependent phenotypes. To test this and to ascertain what functions of Gpx3 can be shared by cGPx-like enzymes, we constructed a novel cGPx-like yeast enzyme – cGpx3. We confirmed that the ‘gap’ sequences conserved among cGPxs but absent from aligned PHGPx sequences are the principal cause of these enzymes’ structural and functional differences. Peroxidase activity against a cGPx substrate was high in the cGpx3 construct, which was multimeric and had a peroxidase catalytic mechanism distinct from Gpx3, but cGpx3 was defective for phospholipid hydroperoxidase and signaling activities. cGpx3 did not complement the sensitivity to lipid peroxidation of a gpxΔ mutant, and the resistance to lipid peroxidation conferred by Gpx3 was independent of Yap1, establishing a functional role for Gpx3 phospholipid hydroperoxidase activity. Using the comparison between cGpx3 and Gpx3 in conjunction with other constructs to probe lipid peroxidation as a toxicity mechanism, we also ascertained that lipid peroxidation-dependent processes are a principal cause of cellular cadmium toxicity. The results demonstrate that phospholipid hydroperoxidase and Yap1-mediated signaling activities of Gpx3 have independent functional roles, though both functions depend on the absence of cGPx-like subunit interaction sites, and they resolve more-clearly the potential drivers of differential selective evolution of GPx-like enzymes.
All aerobic organisms require protection from reactive oxygen species (ROS), which are generated continually as by-products of cellular metabolism. Such protection is especially important during exposure to stressors that may promote ROS production, such as pro-oxidants, metals and other xenobiotics (1, 2). Thus, organisms express an array of non-enzymatic and enzymatic antioxidant defense mechanisms. Peroxidases such as catalases, glutathione peroxidases (GPxs) and other thiol-dependent peroxidases are important enzymatic defense systems that reduce hydroperoxides (e.g., tert-butyl hydroperoxide, H₂O₂). The GPxs are commonly considered the most important for ROS defense since they have broader substrate specificities and stronger affinity for H₂O₂ than catalases (1, 3).

Two of the main types of GPx that have been characterized in cells are the classical GPx (cGPx) and the phospholipid hydroperoxide GPx (PHGPx). cGPx is multimeric (commonly tetrameric) and soluble, whereas PHGPx is monomeric and often membrane-associated (4). Diverse functions in signaling and cellular differentiation (including spermatogenesis) have been reported for PHGPxs, but not cGPxs (3, 5). In addition, whereas cGPx has specificity for glutathione as a reducing substrate, PHGPx re-reduction may be coupled to alternative systems such as the thioredoxin pathway (5-7). Moreover, as well as the inorganic and organic hydroperoxides that are substrates of both proteins’ enzymatic activities, PHGPx alone reduces lipid hydroperoxides esterified to biomembranes. Thus, PHGPx is often considered the principal cellular enzyme capable of repairing membrane lipid peroxidation, the highly-damaging process that has been linked to pathological conditions such as ischemic injury, atherosclerosis, and carcinogenesis (2). However, it has been questioned increasingly in recent years whether the phospholipid hydroperoxidase activity is the physiologically-relevant function of PHGPx in vivo, or whether more diversified roles may be of greater importance (3, 8).
PHGPx is less ubiquitous than cGPx in higher eukaryotes (9). In contrast, the yeast Saccharomyces cerevisiae, which is widely used for studying cellular responses to ROS, expresses three GPX genes (10) all of which were found to encode only PHGPx-like proteins, Gpx1, Gpx2 and Gpx3 (11). The latter conclusion was based on protein-sequence comparisons, on analyses of the substrate specificities of the purified GPX1-3 products, and on the susceptibilities to lipid peroxidation (arising from linolenic acid supplementation) of gpx3Δ and gpx1Δ/gpx2Δ/gpx3Δ S. cerevisiae mutants. Further studies have underscored the central role of GPx-like function in antioxidant defense of yeast. It has been established that purified yeast glutaredoxins (Grxs) also have high GPx activity (12), although this seems likely to make only a small contribution to total GPx activity in yeast under standard growth conditions (10, 11). Moreover, Delaunay et al. (5) identified a novel role for yeast Gpx3 (which they term Orp1) as a sensor and transducer of the stress response to hydrogen peroxide. This occurs via Gpx3-mediated oxidation of the Yap1 transcription factor: the proposed model involves sensing of high H2O2 concentrations at the Cys36 residue of Gpx3, which forms a disulfide-linked intermolecular complex with Cys598 in Yap1 which, after rearrangement of the disulfide bond, resolves to yield Yap1(Cys303-Cys598) and re-reduced Gpx3. Yap1 may alternatively be activated by thiol reactive chemicals via a Gpx3-independent pathway (13). It was shown that Gpx3-dependent resistance to H2O2 was due to its signaling function and not to its peroxidase activity as suggested previously (5). The role of the redox-transducing function in protection against phospholipid hydroperoxides was not tested in those studies.

The expression of three PHGPx-like enzymes combined with the absence of a cGPx is to our knowledge unique to S. cerevisiae. This finding was proposed to indicate that S. cerevisiae may have been under a particular selective pressure to evolve and sustain a high capacity for enzymatic repair of membrane lipid peroxidation specifically (11). The alternative function(s) of Gpx3 outlined above has now raised additional possible explanations for the concentration of PHGPx- versus cGPx-like activity in S. cerevisiae, though it has not yet been tested whether Gpx3-mediated signaling via Yap1
has the potential to be undertaken by a cGPx-like protein. Another important and related question is whether the latter activity of Gpx3 accounts entirely for Gpx3-dependent phenotypes in cells, including resistance to lipid peroxidation (5, 14), or does the phospholipid hydroperoxidase activity of Gpx3 (and other PHGPx-like enzymes) have a functional role?

To help resolve these issues and gain further insight into the role of PHGPx versus cGPx activity in cells, here we constructed a novel cGPx-like protein derived from yeast Gpx3. We confirmed the hypothesis (15) that the ‘gap’ sequences conserved among cGPx enzymes but absent from aligned PHGPx sequences distinguish these groups of enzymes structurally and functionally. The phospholipid hydroperoxide reductase and Yap1-mediated signaling activities of Gpx3 were shown to have independent functional roles, which were both lost in the cGPx-like derivative despite an elevated general (non-phospholipid) hydroperoxidase activity. Finally, the genetic uncoupling of phospholipid hydroperoxidase activity from other Gpx3 functions allowed us to show the importance of that activity in protecting cells against lipid peroxidation, and also against toxicants such as cadmium for which a lipid peroxidation-dependent mode of action was established.
EXPERIMENTAL PROCEDURES

Yeast Strains and Preparation of Yeast Cell Extracts — *Saccharomyces cerevisiae* BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and derivative deletion-mutants gpx1Δ and yap1Δ were obtained from Euroscarf (Frankfurt, Germany). An isogenic gpx1Δ/gpx2Δ/gpx3Δ triple mutant was constructed previously (11). A yap1Δ/gpx3Δ double mutant in the BY4741 background was constructed by short flanking homology (SFH) PCR (16): the URA3 marker was used to disrupt GPX3 in the yap1Δ deletion strain from Euroscarf (primer sequences are available on request). A gpx1Δ/gpx2Δ/gpx3Δ (gpx1::URA3, gpx2::HIS3, gpx3::KanMX4) triple mutant derived from *S. cerevisiae* S150-2B (MATa his3Δ1 leu2-3,112 trp1-289 ura3-52) was constructed in this study, as outlined previously for BY4741 (11). *S. cerevisiae* YPH98 (MATa leu2-Δ1 trp1-Δ1 ura3-52 lys2-801amber ade2-101ochre) and the isogenic derivative gpx3Δ, in addition to the vector pRS316-HA-Gpx3C82S (5), were kindly provided by M. Toledano (CNRS, France). Yeast cell extracts were prepared as described previously (11).

Construction of cGpx3 — Splice over-extension PCR (19) was used to introduce the putative subunit-interaction sequences (which are well conserved in cGPxs) of human cGPx1 into yeast Gpx3, using pET14b-GPX3 (11) as the plasmid template. To insert coding sequences for the amino acid sequence LNSLKYVRPGGFEPN in place of AQFCQLNYGVT from Gpx3 (Fig. 1A), a ~5.6 Kb DNA fragment was amplified using KOD DNA polymerase (Novagen) with the forward and reverse primers:

5’- ATGTTAGACCAGGTGTTTCCAACAAATTATCCCATATGAAAAAATTGACG-3’

and

5’- ACCACCTGGTTCAACATATTTCAAAAGAATTCAAAAAATTTTCTTCTCCATCAGAGCCAGGTTTCTTG-3’.

Purified PCR products were digested overnight with *Dpn*I (NEB) at 37°C to remove all
methylated DNA. Competent *E. coli* (Top 10) were transformed directly with the digestion mix. Colony-direct PCR (20) with *GPX3*-specific primers was used to detect appropriately-modified plasmids in transformants which gave a PCR product of 514 bp, versus 499 bp with the unmodified plasmid (a 100-bp ladder from NEB was used as marker). Sequence was verified by automatic DNA sequencing. Modified plasmids were then used as the template to insert coding sequences for the second amino acid sequence of interest. Here the sequence REALPAPSDDATALMTDPKLITWSPVCRNDVA was used to replace the sequence KSQKSGMLGLRGIK (Fig. 1A). The primers were 5′-GATGACTGATCCAAAATTGATTACTTTGCTCCAGTTTTGAGAAATGATGTGCATGGAA TTTGAAAAATTCTTAGGATCGATAAAAAGGG-3′ and 5′-ATCAATTGGATCATCCAGTTGCATCATCATCATGATGTGGGTGGCCAATGCTTCT CTCAAAAAACTTGAAACAGGGTCCTCATTGCC -3′. All other steps were as described above for insertion of the first sequence of interest; a 568-bp PCR product indicated appropriately modified plasmids in this case.

*Gpx3p and cGpx3p Expression and Purification from* *Escherichia coli* — The plasmids pET14b-*GPX3* and pET14b-*cGPX3* (above) were used for expression of His-tagged Gpx3 and cGPx3 in *Escherichia coli* BL21 (DE3, Promega), as we have described previously (11). The His-tagged proteins were purified from *E. coli* cell extracts according to the manufacturer’s instructions using 1 mL HiTrap chelating affinity columns (Amersham Pharmacia Biotech), charged with 0.1 M NiSO₄. The procedures were as described previously (11). Fractions containing the protein peaks (18) were assayed immediately for enzymatic activity. Soluble recombinant protein was also analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) after affinity purification using standard protocols (20).
Gel Filtration Chromatography — Gel filtration chromatography was used to estimate the molecular weights of the native Gpx3 and cGpx3 proteins in their active states. A commercial GPx (Bovine cGPx, Sigma) was also analyzed for comparison. Fractions that contained peak activities from affinity purification (above) were pooled and passed through a desalting column (5 ml; Amersham Pharmacia Biotechnology). Proteins were eluted with 0.02 M sodium phosphate, pH 7.4. The eluate (~2 ml) was collected and applied to a sephacryl S-200 gel filtration column (Amersham Pharmacia Biotechnology) equilibrated with 0.02 M sodium phosphate, pH 7.4. Protein was eluted at 0.5 ml min⁻¹ and collected in 1 ml fractions. Protein concentrations in the fractions were determined (18), and the molecular weights of the proteins were determined by comparison to standards (MW-GF-200, molecular weight range 12,000–200,000 Da; Sigma).

Gpx3p and cGpx3p Expression in S. cerevisiae — GPX3 was cloned into the multicopy yeast episomal vector YEp351 using the Gateway Clonase system (Invitrogen). Briefly, a fragment comprising the GPX3 open reading frame together with 959 bp of upstream promoter sequence was amplified from yeast genomic DNA with PCR, incorporating primer-encoded AttB sites near the 5’ termini of the PCR product (primer sequences are available on request). The PCR product was cloned into the vector pDONR using the BP clonase enzyme mix (Invitrogen) to produce an ‘Entry’ vector. The RfB cassette containing AttR sites (Invitrogen) was ligated into the Smal site of YEp351 to produce a ‘Destination’ vector. The GPX3 fragment was cloned from the Entry vector into the Destination vector using the LR clonase enzyme mix (Invitrogen), creating YEp351-Gpx3. YEp351-cGpx3 was constructed by splice over-extension PCR, using exactly the same procedures as described under ‘Construction of cGpx3’ but with YEp351-cGpx3 as the plasmid template. Appropriate sequence for all modified plasmids was confirmed.
by automated DNA sequencing. Plasmids were transformed into S. cerevisiae using the lithium acetate method (21) and transformants selected on YNB medium with appropriate supplements.

**Enzyme Assays** — Phospholipid hydroperoxidase activity *in vitro* was determined spectrophotometrically according to NADPH oxidation, which is coupled with the reduction of phosphatidylcholine hydroperoxide to the corresponding hydroxyphospholipid (9, 11). Glutathione (GSH) was favored over thioredoxin (5) as the reducing system in this study since GSH but not thioredoxin serves as a substrate for both PHGPx- and cGPx-like enzymes, allowing direct comparison of activities. GSH-dependent GPx activity in yeast extracts has been reported in several studies (10, 11, 22), but the sensitivity of detection is relatively low, so relatively large amounts of protein are included in assays (11). Phosphatidylcholine hydroperoxide (PCOOH) was generated and purified as described previously (11). Enzymatic activities in crude cell extracts or of purified recombinant protein, with either PCOOH or t-BHP substrates, were assayed exactly as described previously (11).

β-Galactosidase activity was assayed in *S. cerevisiae* cells transformed with the vector pyTRX2 (a gift from D. Jamieson, Heriot-Watt University), in which the lacZ structural gene is expressed under the control of the Yap1-responsive TRX2 promoter of *S. cerevisiae* (23). Transformants were selected in YNB–Leu medium and grown to exponential phase (\(A_{600} \approx 1.0\)). β-Galactosidase activity was determined (20) before and at intervals after exposing cells to 0.4 mM H\(_2\)O\(_2\).

**Phenotype analyses** — Organisms were cultured to mid-/late-exponential phase (\(A_{600} \approx 2.0\)) either in liquid YEPD medium or, for experiments involving plasmid-bearing strains, in YNB medium supplemented with the appropriate amino acids or nucleic acid bases (20). For spotting experiments with H\(_2\)O\(_2\) or Cd, the cultures were adjusted to \(A_{600}\) of ~1, 0.1, 0.01, 0.001 and 0.0001 for each strain. Samples (5 \(\mu\)l) from each dilution were spotted on YEPD agar, supplemented with
H₂O₂ or Cd(NO₃)₂ as specified. For all experiments involving linolenic acid (18:3), media were prepared with tergitol to solubilize the fatty acid and 1 µl aliquots were spotted from dilution series starting at OD₆₀₀ ~5.0. The final tergitol concentration was 1% (wt/vol); tergitol has no adverse effect on yeast growth (17). Growth was examined after incubation for 4 d at 30ºC.
RESULTS

The Insertion of Putative cGPx Subunit-Interaction Sequences to Gpx3 yields a cGPx-Like Protein — Sequence alignments of PHGPx proteins against cGPxs produce two characteristic sequence gaps in the PHGPxs (11, 15). These gaps correspond closely to the predicted subunit interaction sites of cGPx enzymes (15), which form α-helix and loop structures that are incomplete or absent in PHGPx-like enzymes (4). Consequently, it was proposed that the gaps at these sites might dictate the monomeric nature and the substrate range of PHGPxs (15). We tested this hypothesis by inserting the subunit interaction sequences from human cGPx (hGPx1) at the corresponding gap positions of Gpx3 (Fig. 1A), the principal phospholipid hydroperoxidase of *S. cerevisiae* (11). The inserted sequences were codon-optimized for yeast, and were the minimum fragment-lengths estimated to be necessary for introducing to Gpx3 the conserved cGPx α-helix and loop structures (4). Appropriate modification of the *GPX3* open reading frame was confirmed with PCR and automatic DNA sequencing. Analysis of the wild type and modified proteins by denaturing PAGE, after heterologous expression in *E. coli* and affinity purification, confirmed that the inserted sequences were incorporated into the novel full-length protein product, which was termed cGpx3 (Fig. 1B). Size-exclusion chromatography under non-denaturing conditions was used to estimate the native molecular weights of Gpx3 and cGpx3 expressed in *E. coli*. The purified proteins could be readily discriminated according to size under these conditions (Fig. 1C). By comparison with protein standards, the molecular weights of native Gpx3 and cGpx3 were calculated as ~22 kDa and ~49 kDa, respectively. Compared to the molecular weights obtained with denaturing-gel electrophoresis (see Fig. 1B), these results corresponded to a multimeric (dimeric) structure for cGpx3, in contrast to Gpx3 which is monomeric (11). All documented cGPx-like enzymes from other organisms form dimers or tetramers (4, 24, 25). As a positive control, we confirmed the tetrameric structure of a
commercially-available bovine cGPx according its molecular weight (~80kDa), using the same procedures as above.

Initial enzymatic assays with proteins induced heterologously in *E. coli* revealed that purified cGpx3 had an approximate 7-fold higher relative activity with a generic GPx substrate (*t*-BHP) than a PHGPx-specific substrate (PCOOH) compared to purified Gpx3 (data not shown). Moreover, the detection of strong peroxidase activity with cGpx3 was in spite of the replacement of Cys82, a residue essential for the peroxidase activity of Gpx3 (5). This was consistent with a peroxidase catalytic mechanism in cGpx3 similar to that of other cGpxs and distinct from Gpx3 (see Discussion). This prompted us to substantiate the above results by comparing the enzymatic activities under more native conditions, i.e. in *S. cerevisiae* and expressed under the control of the *GPX3* promoter. Most of the analyses were with cells overexpressing Gpx3 or cGpx3 from multi-copy plasmids, in order to maximize the sensitivity of detection of any cGpx3-dependent phenotypes (see later) and phospholipid hydroperoxidase activity. Consistent with data for the heterologously-expressed proteins (above), protein extracts from *S. cerevisiae* *gpx1Δ/gpx2Δ/gpx3Δ* cells that were transformed to express only cGpx3 exhibited a ~2-fold higher specific activity against the GPx substrate *t*-BHP than extracts from Gpx3-expressing cells (Fig. 2). This high general (non-phospholipid) hydroperoxidase activity of cGpx3 contrasted with a markedly (~5.7-fold) lower specific activity in cGpx3-expressing cells compared to Gpx3-expressing cells when the PHGPx substrate PCOOH was used for assays (Fig. 2). Note that this difference actually underestimates that attributable solely to cGpx3 versus Gpx3 because there is a low background (~10-15% of total) (PH)GPx activity in *gpx1Δ/gpx2Δ/gpx3Δ* cells (10, 11). The results show that insertion of consensus cGPx subunit interaction sequences to yeast Gpx3 created a cGPx-like multimeric protein with cGPx-like substrate specificity.
Loss of Phospholipid Hydroperoxidase Activity is Associated with Loss of Cellular Resistance to Lipid Peroxidation — Resistance to lipid peroxidation conferred by Gpx3 (11) could be due to the enzyme’s general peroxidase activity, or to its phospholipid hydroperoxidase activity specifically, or to its redox-transducing activity. To resolve these possibilities, cGpx3- or Gpx3-expressing cells and a Yap1-deficient mutant were treated with the PUFA linolenic acid (18:3), which is known to inhibit the growth of gpx1Δ/gpx2Δ/gpx3Δ cells in a lipid peroxidation-dependent manner (11, 17). To test whether GPX-dependent resistance to 18:3 might be due to the Yap1-dependent redox-transducing activity of Gpx3, we compared the 18:3 resistances of gpx3Δ and yap1Δ cells. As reported previously (11), gpx3Δ cells exhibited an 18:3-sensitivity phenotype (Fig. 3A). In contrast, yap1Δ cells were 18:3-resistant (and a gpx3Δ/yap1Δ double mutant showed the same phenotype as gpx3Δ cells; not shown). Gpx3-mediated oxidation of the Yap1 transcription factor is responsible for Gpx3-dependent H2O2 resistance (5). Consistent with that conclusion, we confirmed relative sensitivities of gpx3Δ and yap1Δ cells to H2O2 similar to those reported by Delaunay et al. (5) (Fig. 3A). The 18:3-resistance of S. cerevisiae defective for Yap1 shows that, unlike for H2O2, Gpx3-mediated 18:3 resistance can not require the Yap1-dependent signaling pathway.

Cells expressing cGpx3 or Gpx3 were compared to distinguish the contributions of non-phospholipid from phospholipid hydroperoxidase activities to 18:3 resistance. Expression of wild type Gpx3 partially complemented the 18:3-sensitivity phenotype of S. cerevisiae gpx1Δ/gpx2Δ/gpx3Δ (Fig. 3B); the absence of GPX1 and GPX2 apparently precluded full restoration of wild type levels of 18:3-resistance. Moreover, cGpx3 had no discernible impact on the 18:3-hypersensitivity of gpx1Δ/gpx2Δ/gpx3Δ cells, even when cGpx3 was overexpressed (we routinely confirmed that (PH)GPx activities in transformed cells used for experiments were similar to those shown in Fig. 2). Thus, the loss of phospholipid hydroperoxidase-specific activity from Gpx3 (i.e.,
in cGpx3) was coincident with loss of cellular resistance to lipid peroxidation-dependent 18:3 toxicity.

*cGpx3 is Defective for Redox-Transducing Activity* — Since H$_2$O$_2$ resistance conferred by Gpx3 is due to the redox-transducing function of the enzyme rather than its peroxidase activity (5), the differing peroxidase substrate-specificities of Gpx3 and cGpx3 described above were not themselves expected to influence H$_2$O$_2$ resistance. As expected, *S. cerevisiae gpx1Δ/gpx2Δ/gpx3Δ* exhibited a H$_2$O$_2$-sensitivity phenotype that was complemented by expression of Gpx3 (Fig. 4A). However, cGpx3-expressing cells were found to be H$_2$O$_2$-sensitive, as cGpx3 had no influence on the triple-mutant phenotype even when overexpressed. The results suggested that redox-transducing activity was absent in cGpx3. To test this further the response to H$_2$O$_2$ was examined in cells transformed with the plasmid pyTRX2, in which the *lacZ* structural gene is expressed under the control of the Gpx3/Yap1-responsive *TRX2* promoter (23). Consistent with previous results (5, 23), *TRX2* transcription was induced by H$_2$O$_2$ in Gpx3-expressing cells, as reflected by the ~4-fold increase in measured LacZ activity ≥15 min after H$_2$O$_2$ addition (Fig. 4B). In contrast, cGpx3-expressing cells were defective for *TRX2* induction: there was no discernible increase in LacZ activity during the time course of the assay, even when cGpx3 was overexpressed. Thus, cGPx-like properties (e.g., multimericity, and the associated high relative non-phospholipid hydroperoxidase activity) were incompatible not only with phospholipid hydroperoxidase activity (above), but also with the redox-transducing activity of Gpx3.

*Phospholipid Hydroperoxidase Activity Confers Cadmium Resistance in Yeast*— According to the literature, no genetic tool is presently available with which the role of lipid peroxidation in the mode of action of a toxic agent can be tested specifically. We reasoned that cGpx3 and Gpx3 used in conjunction could provide such a tool for establishing the dependency of a phenotype on lipid peroxidation. To test this, resistance to the toxic metal cadmium was selected as a model phenotype,
since previous physiological evidence has suggested a role for lipid peroxidation in Cd toxicity towards yeasts (17, 26). Consistent with that conclusion, we found here that *S. cerevisiae* gpx3Δ single- and gpx1Δ/gpx2Δ/gpx3Δ triple-mutants exhibited cadmium sensitivity phenotypes (Fig. 5B). This result was reproducible in both the *S. cerevisiae* BY4741 and S150-2B backgrounds, though not in a gpx3Δ mutant derived from *S. cerevisiae* YPH98 (not shown) in agreement with a previous study using the same strain (27) [we did not further investigate this discrepancy here since it was not necessary for our purposes (below), but the observation is reminiscent of other strain background-specific stress response phenotypes in *S. cerevisiae* (28)]. Overexpression of GPX3 in *S. cerevisiae* BY4741 resulted in a slight elevation of Cd resistance compared to wild type cells, corroborating a role for Gpx3 in cellular protection from Cd (Fig. 5B). In agreement with previous reports (13, 27), *S. cerevisiae* yap1Δ cells were also Cd-sensitive (Fig. 5A,B). However, the yap1Δ phenotype was markedly stronger than that of gpx3Δ cells (Fig. 5A), a result consistent with recent evidence that Yap1-dependent Cd resistance relies on a molecular redox center in Yap1 which is distinct from that linked to the redox-transducing activity of Gpx3 (13). To substantiate this, gpx3Δ cells were transformed with a single-copy plasmid expressing a Gpx3C82S mutant protein that is defective for peroxidase activity but not for Yap1-mediated signaling activity of Gpx3 (5). Expression of Gpx3C82S did not complement the Cd-sensitivity of the gpx3Δ strain (Fig. 5B), corroborating that a peroxidase activity of Gpx3 is required for Cd resistance. To discern whether this peroxidase activity was phospholipid hydroperoxidase specifically, the Cd resistance of gpx1Δ/gpx2Δ/gpx3Δ cells transformed with plasmids expressing either Gpx3 or cGpx3 was compared. Expression of Gpx3 complemented the Cd-sensitivity of the triple mutant but expression of cGpx3p did not (Fig. 5C). Therefore, with the exclusion of redox-transducing activity as a cause of Gpx3-dependent Cd resistance (above), the loss of phospholipid hydroperoxidase activity specifically from Gpx3 (i.e., in cGpx3) was associated with the loss of cellular resistance to Cd.
DISCUSSION

It has been questioned increasingly in recent years whether the hydroperoxidase activity of PHGPx-like enzymes plays an important functional role in organisms, or whether such activity may be inconsequential relative to the regulatory and other functions that PHGPxs are now known to drive (3, 6). With the recent finding that Gpx3 in *S. cerevisiae* serves as a sensor and transducer of hydrogen peroxide stress, it was speculated that this activity might account for most or all Gpx3-dependent phenotypes, though it was accepted that an important question remained with regard to resistance to lipid peroxides (5, 14). Using novel constructs to discriminate the various activities of yeast Gpx3, the present results indicate that a hydroperoxidase activity of Gpx3 is indeed that which protects cells from lipid peroxidation, and that this role is readily discernible at the phenotypic level. This evidence now undermines the point that, to date, no physiological hydroperoxide detoxification function had been demonstrated for a PHGPx-like enzyme *in vivo* (8). The results also reveal that, despite their lower peroxidase rate constants than the seleno-peroxidases (3, 4), sulfur- (cysteine-) catalyzed peroxidases can have biologically-relevant peroxidase activity. Moreover, our results indicate that it is phospholipid hydroperoxidase activity specifically of a PHGPx-like protein that confers resistance to lipid peroxidation. It had been suggested previously that the non-phospholipid peroxidase activity of (PH)GPx-like enzymes might be more relevant to preventing lipid peroxidation *in vivo* (by diminishing hydroperoxide-dependent initiation of lipid peroxidation) than the phospholipid hydroperoxidase activity (8). Here, the lack of protection against PUFA-mediated lipid peroxidation (11, 17) conferred by cGpx3 was despite the enzyme’s high relative non-phospholipid peroxidase activity. Low-level phospholipid hydroperoxidase activity was detected in cGpx3-expressing cells (Fig. 2) but this, as a relative proportion of the equivalent activity due to Gpx3 under physiological conditions, would be an overestimate (see Results) and evidently too low to have a functional role.
The high non-phospholipid peroxidase activity attributed to cGPx3 was despite the replacement of the Cys82 residue of Gpx3 (see Fig. 1A). Cys82 is reported to be essential for the unusual peroxidase catalytic mechanism of Gpx3, which involves intra-molecular disulfide bond formation (5). Thus, a catalytic system distinct from that of Gpx3 operates in cGPx3. The most likely mechanism would be that described for other cGPx-like enzymes, involving oxidation by hydroperoxides only of the active site (seleno)cysteine (Cys36 in this case) and its reduction by GSH (4). However, the possibility that an alternative intra-molecular disulfide bond forms between Cys36 and the Cys142 residue introduced to cGpx3 (Fig. 1A) cannot be discounted. Moreover, despite its higher relative non-phospholipid peroxidase activity, no functional role for cGpx3 was detected in this study. For example, consistent with the findings of Delaunay et al. (5), the peroxidase activity of neither Gpx3 nor cGpx3 conferred resistance to H2O2. While it is possible that this finding may not hold for all hydroperoxide stresses, the apparent redundancy of this activity suggests that the only biologically-relevant peroxidase activity of non-selenium GPxs may be that directed against phospholipid hydroperoxides by the PHGPx-like enzymes.

The dissection of phospholipid hydroperoxidase activity from other Gpx3 functions provided a novel tool for genetically testing lipid peroxidation as the mechanism of cadmium toxicity. The harmful biological consequences of high Cd doses are widely documented, but the cause of cellular Cd toxicity has not been firmly established. Previous correlation-based studies have indicated that, as well as other possible causes (29), oxidative mechanisms and Cd-induced lipid peroxidation in particular may underlie Cd toxicity (for review, see Ref. 30). For example, antioxidants protect against Cd toxicity (31), and PUFA-enriched cells are Cd-sensitive (17). Oxidative Cd toxicity could involve depletion of cellular GSH, or inhibition of antioxidant defense enzymes (30). Our demonstration that the phospholipid hydroperoxidase activity of Gpx3, specifically, is associated with protection against Cd establishes a likely major role for lipid peroxidation in the mechanism of Cd toxicity. Moreso, since GPX3 overexpression was effective in raising the threshold of Cd
tolerance: whereas gene deletion can lower the threshold of resistance to an agent by sensitizing a new principal cellular target to that agent, only genes that help to protect the normal target(s) of toxicity (or that directly encode that target) should raise the lower resistance-threshold. The conclusion that Gpx3-mediated Cd resistance was independent of Yap1, specifically, was further supported by the fact that the \textit{GPX1} and \textit{GPX2} gene products also contributed to cellular Cd resistance: the \textit{gpx1Δ/gpx2Δ/gpx3Δ} triple mutant was more sensitive to Cd than the \textit{gpx3Δ} single mutant (Fig. 5B). In common with Gpx3, Gpx1 and Gpx2 possess phospholipid hydroperoxidase activity (11) but, unlike Gpx3, are not known to interact with Yap1. Our evidence is consistent with the recent finding that Yap1 is activated by Cd and other thiol reactive chemicals through a Gpx3-independent mechanism (13). Recent work has indicated that Gpx3 also confers aluminium resistance independently of Yap1 in yeast (32). The (c)Gpx3 constructs described here provide a novel genetic tool, which may find general application for testing lipid peroxidation as the mode of action of toxic agents.

The results also confirmed experimentally the hypothesis that the putative subunit interaction sequences of cGPxs (15) indeed differentiate PHGPx- from cGpx-like structure and function. The apparent dimeric structure of cGpx3 was consistent with successful introduction of the loop dimerization interface (4) to Gpx3. However, introduction of the putative \(\alpha\)-helix tetramerization interface (4) alone appeared to be insufficient for tetramerization. Tetramerization evidently was not required for the cGpx-like function that was sought here. Nevertheless, should it be of interest in the future, the present effort provides a start point from which the minimum sequence of human cGpx (the template for the modifications performed here) required for tetramerization could be elucidated precisely. Dimeric and functional GPx is not unique to the present study (24, 25) and can be an obligate property of certain thiol peroxidases, e.g. the 2-cys peroxiredoxins (33).
Delaunay et al. (5) demonstrated that, among the Cys residues of Gpx3, only Cys36 is required for forming the disulfide-linked intermolecular complex with Yap1-Cys598, a reaction that underpins the redox-transducing function of Gpx3. Even though Cys36 and surrounding residues were preserved in cGpx3, the redox-transducing function was not. Rather, the observed defects in Yap1-mediated H$_2$O$_2$ resistance and H$_2$O$_2$-dependent TRX2 induction in cGpx3-expressing cells probably reflect a problem of Yap1 accessibility to the cGpx3 active site. The more ‘opened’ structure of monomeric PHGPx-like enzymes compared to multimeric GPxs is widely considered to be the factor that may allow the monomeric enzymes to react with thiols in bulky molecules (e.g., Yap1) rather than just GSH (4-6). It seems likely that this structure may dictate that signaling (e.g., Yap1-mediated) and other diversified functions of PHGPx-like enzymes cannot be shared by cGPxs of other organisms either.

The likely fitness benefits for yeast associated with preservation of Gpx3-dependent transduction of the H$_2$O$_2$ signal (5) have provided an alternative possible explanation for the apparent evolutionary selection of PHGPx- over GPx-like activity in S. cerevisiae. Previously, it was proposed that the concentration of PHGPx-like activities in yeast reflected a selective pressure to evolve and sustain a high capacity for enzymatic repair of membrane lipid peroxidation specifically (11). Certainly, the GPX3 gene occurs in a region of chromosome IX of S. cerevisiae that appears particularly prone to chromosomal rearrangements, and selective amplification of this and certain similar regions is considered to play a major role in adaptive evolution (34). However, while pressure to preserve redox-transducing activity could be a factor in the selective retention or amplification of Gpx3, the same is not true of Gpx1 and Gpx2 which have not been found to share this activity (11). Some other functional diversification is apparent in Gpx1 and Gpx2. For example, unlike GPX3, GPX1 and GPX2 are induced transcriptionally by glucose starvation and oxidative stress, respectively (10) and Gpx2 also appears to play a role in the response to Ca$^{2+}$ (35). Moreover,
the principal feature common to all three enzymes is hydroperoxidase activity and – from a functional perspective according to this and our previous study (11) – phospholipid hydroperoxidase activity specifically. Thus, in an evolutionary context, the expression of Gpx1, Gpx2 and Gpx3 [in conjunction with the lack of PUFA synthesis (11, 17)] would be adaptations expected of an organism that may be prone to particularly high ROS fluxes generated in or around membranes (11). It is not known whether *S. cerevisiae* membranes are especially susceptible to oxidative attack, though we have noted from recent searches one novel potential explanation for any such susceptibility. *S. cerevisiae* lacks the alternative oxidase that is used for cyanide-resistant respiration (CRR) in other organisms (36). CRR is widely considered to enable organisms to respire while evading the excess ROS generation that can arise during normal respiration, and may be particularly helpful for averting ROS-dependent lipid peroxidation (37). We have noted that the distribution of CRR among most organisms (including different yeasts) matches that of PUFA synthetic ability almost exactly, i.e. very few organisms exhibit one of these functions without the other. It is tempting to speculate that in *S. cerevisiae*, perhaps in addition to the absence of PUFA synthesis, the expression of three PHGPx-like enzymes could serve to compensate for the lack of a CRR pathway in defense against lipid peroxidation – a possibility that warrants investigation.

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FIG. 1. **Construction of a cGPx from yeast GPx3.**  
A, Yeast codon-optimized oligonucleotides encoding the putative subunit-interaction sequences from human cGPx1 (dark background) were inserted into the GPx3-encoding yeast GPX3 gene by splice over-extension PCR as outlined under ‘Experimental Procedures’, to create cGPX3 (only the relevant portions of the protein sequences are shown).  
B, Separation by SDS-polyacrylamide gel electrophoresis of affinity-purified His-tagged Gpx3 and cGpx3 after overexpression in *E. coli*.  
C, Overlays of elution profiles for purified Gpx3 and cGpx3, obtained using non-denaturing gel filtration chromatography; the molecular weights of the native proteins were calculated by comparison to standards (12,000–200,000 Da).

FIG. 2. **Substrate-specific activities of Gpx3 and cGpx3 expressed in *S. cerevisiae*.** Enzyme activities were assayed in extracts from *S. cerevisiae gpx1Δ/gpx2Δ/gpx3Δ* cells either transformed or not with YEp351-Gpx3 (‘+Gpx3’) or YEp351-cGpx3 (‘+cGpx3’), as outlined under ‘Experimental Procedures’. The *gpx1Δ/gpx2Δ/gpx3Δ* triple-mutant background was used to remove activity arising from chromosomal *GPX* genes. Assays were performed using either *t*-BHP (□) or PCOOH (■) as the hydroperoxide substrate. The data shown are means from three independent assays ± S.D. Similar results were obtained with affinity-purified Gpx3 and cGpx3 expressed heterologously in *E. coli*, and in another *S. cerevisiae* strain background (BY4741) (not shown).

FIG. 3. **Loss of phospholipid hydroperoxidase activity is associated with loss of cellular resistance to linolenic acid-mediated lipid peroxidation.** *S. cerevisiae* BY4741 and the isogenic mutant strains shown were harvested during the exponential phase of growth and dilutions of
decreasing concentration were spotted from left to right on each agar plate (supplemented as shown) for each strain. Growth was examined after incubation for 4 d at 30°C. A, The wild type (not shown) exhibited no growth defect at 1 mM H₂O₂ or 1 mM linolenic acid. B, ‘+Gpx3’ and ‘+cGpx3’ refer to strains transformed with the plasmids YEp351-Gpx3 and YEp351-cGpx3, respectively. Typical results from one of at least three independent experiments are shown.

FIG. 4. cGpx3 is defective for redox-transducing activity. A. S. cerevisiae gpx1Δ/gpx2Δ/gpx3Δ cells either transformed or not with YEp351-Gpx3 (‘+Gpx3’) or YEp351-cGpx3 (‘+cGpx3’) were harvested during the exponential phase of growth and dilutions of decreasing concentration were spotted from left to right on each agar plate (supplemented or not with 4 mM H₂O₂) for each strain. Growth was examined after incubation for 4 d at 30°C. Typical results from one of three independent experiments are shown. B, The LacZ activity of S. cerevisiae gpx1Δ/gpx2Δ/gpx3Δ co-transformed with pyTRX2 (expressing a TRX2-lacZ fusion) and either YEp351-Gpx3 (○) or YEp351-cGpx3 (●) was monitored before and during exposure to 0.4 mM H₂O₂. Units of LacZ activity were calculated according to (20). The points are means from two independent experiments, ± S.D. where these exceed the dimensions of the symbols. The results shown refer to the S. cerevisiae S150-2B strain background. Similar results were confirmed in the BY4741 background (not shown).

FIG. 5. Phospholipid-hydroperoxidase activity confers cadmium resistance in S. cerevisiae. S. cerevisiae BY4741 and the isogenic mutant strains shown were harvested during the exponential phase of growth and dilutions of decreasing concentration (from an A₆₀₀ ~1.0 to ~0.0001) were inoculated in 5 µl spots from left to right on each plate for each strain. Growth in the presence
of Cd(NO₃)₂ was examined after incubation for 4 d at 30°C. Control growth in the absence of Cd(NO₃)₂ (not shown) was similar for all strains to that shown for the wild type at 30 μM Cd(NO₃)₂ (A). B, ‘+YEp351-Gpx3’ and ‘+Gpx3C82S’, indicates strains transformed with the plasmids YEp351-Gpx3 and pRS316-HA-Gpx3C82S, respectively. The medium was supplemented with 50 μM Cd(NO₃)₂. C, ‘+Gpx3’ and ‘+cGpx3’ indicates strains transformed with the plasmids YEp351-Gpx3 and YEp351-cGpx3, respectively. The medium was supplemented with 40 μM Cd(NO₃)₂. Each panel shows typical results from one of at least three independent experiments.
Figure 2

Specific activity (mU/mg protein)

- $gpx1,2,3\Delta + Gpx3$
- $gpx1,2,3\Delta + cGpx3$
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