The cytosolic thioredoxin peroxidase II (cTPxII/Tsa2p) from *Saccharomyces cerevisiae* shares 86% identity with the relatively well-characterized cytosolic thioredoxin peroxidase I (cTPxI/Tsa1p). In contrast to cTPxI protein, cTPxII is not abundant and is highly inducible by peroxides. Here, we describe a unique phenotype for ΔcTPxII strain; these cells were highly sensitive to tert-butylhydroperoxide (TBHP) but presented resistance to H$_2$O$_2$ in fermentative and respiratory conditions. In contrast, ΔcTPxI strain was very sensitive to both TBHP and H$_2$O$_2$, whatever the carbon source present in the media. These differences in the response of mutant cells to the different kinds of peroxide insult could not be attributed to enzymatic properties of cTPxI and cTPxII since the recombinant proteins showed similar in vitro efficiencies ($K_{cat}/K_m$) in the removals of both kinds of peroxide. This specific sensitivity of ΔcTPxII cells to TBHP could not be related to the expression pattern of *TSA2* (cytosolic thioredoxin peroxidase II gene) either, since this gene is highly inducible by both H$_2$O$_2$ and TBHP when cells were grown in different conditions. Finally, peroxide-removing assays were performed and showed that catalase activity increased significantly only in ΔcTPxII cells, which appear to be related with the resistance of this strain to H$_2$O$_2$. Taken together, present data indicate that cTPxII and cTPxI are key components of the yeast defense system against organic peroxide insult. In regard to the stress induced by H$_2$O$_2$, catalases (peroxisomal and/or cytosolic) and cTPxII seemed to cooperate with cTPxI in the defense of yeast against this oxidant.

Cells possess multiple pathways to detoxify peroxides. In the case of the yeast *Saccharomyces cerevisiae*, there are five peroxiredoxins (a class of thiol-dependent peroxidases) located in different cell compartments; two catalases (one peroxisomal and one cytosolic) and one cytochrome c peroxidase (a mitochondrial heme-dependent protein) (for review see Refs. 1 and 2). In addition, three phospholipid glutathione peroxidases have been recently characterized, although their cellular locations are still unknown (3, 4). It is reasonable to assume that each peroxide-removing enzyme plays a particular role in cell protection against oxidative stress, which can be defined by its cellular location, substrate specificity, and expression pattern among other factors.

In regard to substrate specificity, catalase T (cytosolic) and catalase A (peroxisomal) can only decompose H$_2$O$_2$ (Reaction 1), whereas thiol-dependent peroxidases can reduce both organic hydroperoxide and H$_2$O$_2$ (see Reaction 2 below; when $R^2 = H$, $R^2$OOH = H$_2$O$_2$; when $R^2$ = alkyl; $R^2$OOH = alkyl hydroperoxide (5–7)). Peroxidases can oxidize different substrates such as glutathione and thioredoxin, which can also be relevant for cell signaling since these thiols are involved in modulation of transcriptional factors and other regulators (8–11).

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

*Reaction 1*

$$2R^1SH + R^2OOH \rightarrow R^1SSR^1 + H_2O + R^2OH$$

*Reaction 2*

Yeast has been employed as a model for higher eukaryotes because, among other reasons, it is highly amenable to genetic manipulations, and its genome is fully sequenced. This microorganism is also suitable for oxidative stress studies because it can grow both anaerobically or in the presence of high oxygen tension. Moreover, the kind of carbon source present in the medium provokes dramatic effects on yeast biochemistry and physiology. Through various signaling pathways, glucose represses several genes involved in respiration and in mitochondrial biogenesis (12). The expression of several antioxidant enzymes is also repressed by glucose, but in some cases this effect is only slight (for review, see Refs. 1 and 13). At high glucose levels mitochondria are present with few cristae that are not well developed (14), and as a consequence, this organelle is not very active. On the other side, when glucose is consumed from the culture medium, yeast undergoes great physiological and biochemical changes to produce ATP mainly by oxidative phosphorylation, and consequently, more reactive oxygen species are generated. Therefore, by changing the carbon source in the media, bioenergetics of yeast is profoundly affected.

Expression of peroxiredoxins is also affected by glucose in yeast (15, 16). cTPxI ($^*$ also known as *Tsa1p*) from *S. cerevisiae* was repressed by glucose.

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$^*$ The abbreviations used are: cTPxI, cytosolic thioredoxin peroxidase I protein, also known as Tsa1p (YML028W); cTPxII, cytosolic thioredoxin peroxidase II protein, also known as Tsa2p (YDR453C); cTPxIII, cytosolic thioredoxin peroxidase III, also known as Ahp1, type II TPx; or TSA II (YLR109W); mTPxI, mitochondrial thioredoxin peroxidase I, also known as Prx1 (YBL064C); TBHP, tert-butylhydroperoxide; TRR1, thioredoxin reductase I gene (YDR353W); Trr1, thioredoxin reductase I protein (YDR353W); Trx2, thioredoxin 2 protein (YGR209C); TSA1, cytosolic thioredoxin peroxidase I gene (YML028W); TSA2, cytosolic thioredoxin peroxidase II gene (YDR453C); DTPA, diethylenetriaminepentaacetic acid.

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cTPxI, cTPxII, and Organic Hydroperoxide Detoxification

was the first peroxiredoxin described in a eukaryotic cell and is a very abundant protein (17, 18). Its importance in cell protection against peroxide insult has been demonstrated in several reports (5, 19), especially in cells with dysfunctional mitochondria (15). Furthermore, it was shown in a genome-wide screen that TSA1 has an important role in the protection of yeast against accumulation of mutations and of chromosomal rearrangements (20). cTPxII (also known as Tsa2p) appears to be a duplication of cTPxI, since they share 98% identity in their amino acid sequence, and both are located in the cytosol (2, 21). Contrary to cTPxI, the levels of cTPxII are very low under basal conditions (2, 22). Here, we have analyzed the expression pattern of cTPxII and the viability of ΔcTPxII cells under several conditions, as well as their enzymatic properties of recombinant cTPxII. cTPxII and cTPxI were investigated in detail. Our results indicated that cTPxII and cTPxI, among other antioxidants, are key components of yeast defense against stress induced by organic peroxides independent of the carbon source present in the media. Moreover, biochemical assays performed in cell extracts indicated that catalase cooperate with peroxiredoxin in the protection of yeast against H2O2 insult.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions—S. cerevisiae yeast strains used in this study were BY4741 (Mat a; His3Δ1; Leu2Δ0; Met15Δ0; Ura3Δ0), cTPxI (Mat a; His3Δ1; Leu2Δ0; Lys2Δ0; Ura3Δ0; YML028W::Kan Mx4), and cTPxII (Mat a; His3Δ1; Leu2Δ0; Met15Δ0; Ura3Δ0; YDR653C::Kan Mx4) and were obtained from EUROSCARF (http://www.uni-frankfurt.de/fb15/mikro/euroscarf/index.html).

Cells were grown at 30 °C in complete synthetic medium (23) containing glucose, glycerol, or raffinose as carbon sources. For most analysis, except when expression of the TSA2 gene was tested at different phases of growth, cells were collected at the mid-logarithmic phase, usually at A600 nm = 0.8.

To obtain high amounts of cells adapted to growth under glycerol or raffinose as the carbon source, pre-cultures were grown overnight in glucose, harvested, and transferred to the respective media. Cells were then cultivated for 8 h in glycerol or raffinose-containing medium. This condition was sufficient to induce yeast adaptation from a fermentative to a respiratory condition (16).

Determination of Peroxide Tolerance—Tolerance of yeast cells to H2O2 was determined by the spot test as described below. Inoculates were obtained from cells that were grown overnight in complete synthetic medium with 2% glucose. In the case of the experiments conducted in fermentative conditions, inoculates were diluted to A0.001 nm = 0.2 on the next day. Afterward, yeast was grown until cell density reached a value equivalent to A0.001 nm = 0.8. Finally, cell cultures were diluted again to A0.001 nm = 0.2, and then four subsequent 1:5 dilutions of these cell suspensions were performed. A 10-μl droplet of each dilution was plated onto complete synthetic medium plus agar with glucose 2%.

For experiments in respiratory conditions, inoculates were diluted in synthetic medium containing glycerol with a cell density equivalent to A0.001 nm = 0.8. Cells were then cultivated for 8 h in glycerol-containing medium and then diluted to A0.001 nm = 0.2. Four subsequent 1:5 dilutions of these cell suspensions were performed. A 10-μl droplet of each dilution was plated onto complete synthetic medium plus agar with glucose 2% and glucose 0.1%.

When glucose or glycerol was used as the carbon source, plates were incubated for 30 or 48 h, respectively. Peroxides were added to plates at the concentrations indicated in the figures.

Northern Blot Analysis—All the procedures were according to the membrane manufacturer’s protocol and as described by Ausubel et al. (23). In summary, analyses were conducted on total yeast RNA extracted from cells under distinct growth conditions by hot acid phenol method (15, 16) and separated by electrophoresis on formaldehyde-agarose gels. The fractionated RNAs were transferred to a positively charged nylon membrane (Amersham International) and fixed. Probed membranes were exposed to Kodak films (X-Omat). For probe preparation, a 600-bp NdeI/BamHI fragment containing the TSA2-coding sequence was isolated from plasmid pPROEX/TSA2. Plasmid DNA preparation, gel electrophoresis, and purification were all carried out using standard methods. The resulting purified fragment was used to construct the TSA2-32P-labeled probe by random-primed labeling. Ribosomal RNA, whose abundance was fairly constant under different growth conditions or among strain derivatives, was used as a loading control (15, 16). The amount of RNA in each well was determined by the fluorescence of ethidium bromide bound to this nucleic acid and by nucleic acid absorbance at 260 nm (1 A260 nm = 40 μg/ml RNA).

When Trr1, cTPxI, and cTPxII—TSA2 was PCR-amplified from yeast genomic DNA using the following forward 5′-TATCATATGGTAGCAGAGTCACAAACACAG-GCC-3′ and reverse 5′-TAGGATCCTAAATTATGGCATTITTTTG-3′ primers. The underlined bases represent the NdeI and BamHI sites, respectively. The PCR product was cloned into the pGEM-Teasy vector (Promega), resulting in the insertion of TSA2 into pGEM/TSA2 plasmid. An Escherichia coli DH5-α strain was transformed with pGEM/TSA2, and white colonies were selected from LB-ampicillin-X-gal (X-gal: 5-bromo-4-chloro-3-indolyl-β-p-galactopyranoside) medium. Plasmid extraction was performed using the Rapid Plasmid Miniprep System Concert kit (Invitrogen). pPROEX (Invitrogen) and pGEM/TSA2 were first digested with NdeI and then with BamHI. Both p-GROEX and pGEM/TSA2 digestion products were extracted from an agarose gel using the Rapid Gel Extraction Kit (Invitrogen), and the TSA2 fragment was ligated to the digested pGROEX expression vector. The resulting pPROEX/TSA2 plasmid was sequenced with an Applied Biosystems ABI Prism 3700 apparatus to confirm that the construction was correct. An E. coli DH5-α strain was transformed with p-GROEX/TSA2, and its proteins were subjected to cTPxII expression and purification. Expression vectors for Trr1, Trx1, and cTPxII were similarly constructed after their genes were cloned into NdeI and BamHI restriction sites of pPROEX, pET17b (Novagen), and pET15b (Novagen) expression vectors, respectively.

Protein Expression and Purification—The E. coli DH5-α strain transformed with p-GROEX/TSA2 or with p-GROEX/TRR1 were cultured (50 ml) overnight in LB plus ampicillin medium (100 μg/ml), transferred to 1 liter of fresh LB plus ampicillin medium and cultured until the A600 reached 0.6–0.8. Isopropyl-1-thio-β-galactopyranoside was then added at a final concentration equivalent to 1 mM. After 3 h of incubation, cells were harvested by centrifugation. The pellet was washed and suspended in the start buffer (0.075 M Tris-HCl, pH 7.4, with 500 mM NaCl). Two sonication cycles of 30 s (35% amplitude) followed by 30 s on ice were applied to the cell suspension. The cell extract was then kept on ice during treatment with 1% streptomycin sulfate for 15 min. The suspension was centrifuged at 31,500 × g for 30 min to remove nucleic acid precipitates and cell debris. cTPxI and Trx1 were expressed by similar protocols, but BI21(DES) strain was used as the host instead of DH5-α strain.

Cell extracts containing bacteria that expressed cTPxI, cTPxII, or Trr1 were applied to a Hi-trap nickel-affinity column (Amersham Biosciences) or Talon cobalt-affinity resin (BD Biosciences Clontech, Palo Alto, CA). When Talon cobalt-affinity resin was used, the start buffer was replaced to 50 m Tris-HCl, pH 7.5 with 100 mM NaCl. The conditions for protein purification were optimized using the gradient procedure for imidazole concentration as described by the manufacturer. Trx1 was purified by boiling bacterial extracts as described previously (24).

Determination of Thioredoxin-dependent Peroxidase Activity—Thiol-dependent peroxidase activities of cTPxI and cTPxII were measured by NADPH oxidation assay. NADPH oxidation was monitored at A340 nm (Hitachi Model U-2001 Spectrophotometer) in 1-ml reaction mixtures containing 50 mM Hepes-NaOH, pH 7.4, 100 mM DTPA, 1 mM azide, 0.225 μm Trx2, 0.075 μM Trx1, 2.1 μM cTPxII and 0.18 μM NADPH. The reaction was started by the addition of 10–100 μM peroxide solution, and the mixture was incubated at 30 °C. Protein concentrations were determined by their absorbance at 280 nm. The extinction coefficients for cTPxI (ε290 nm = 22800 M−1 cm−1) and for cTPxII (ε290 nm = 26150 M−1 cm−1) were obtained through ProtParam tool (http://prosite.expasy.org/tools/protparam.html). Ka and Vmax were calculated by the Lineweaver-Burk equation (the double-reciprocal plot of Michaelis-Menten equation). Ka was obtained by dividing Vmax by the peroxiredoxin concentration. No data were included, when Trx1 was observed to give a better fit on the reaction mixture in any of the conditions analyzed.

Determination of Peroxide Consumption by Soluble Cell Extracts—Cells were grown at 30 °C in complete synthetic medium (23) containing 2% glucose. When cells reached a density equal to A600 nm = 0.8, they were harvested by centrifugation at 16,000 × g for 5 min. The pellets were washed in 400 μl of 50 mM Hepes, pH 7.4, 100 mM NaCl buffer containing 2 μg/ml leupeptin and 1 μg/ml pepstatin. Glass beads were added at the same volume of the sample. Two cycles of 6 min of vortex after 6 min on ice were applied to cell suspension. The suspension was centrifuged at 16,000 × g for 5 min. Supernatants were
cTPxI, cTPxII, and Organic Hydroperoxide Detoxification

**Fig. 1.** TBHP tolerance by yeast strains. Tolerance was measured by the spot test as described under “Experimental Procedures.” The first spot on the left in each lane corresponds to the cell suspension diluted to an OD~600~nm~ = ~0.2. Four subsequent 1:5 dilutions of cell suspensions were performed from left to right. Cell dilutions and carbon sources used are described at the top of the figure. Yeast strains and TBHP concentrations used are described at the side of the figure. This figure is representative of at least three similar experiments. WT, wild type.

| Glucose | Glycerol |
|---------|----------|
| Control | Control |
| ΔcTPxI | ΔcTPxI |
| WT | WT |
| ΔcTPxII | ΔcTPxII |
| ΔcTPxI | ΔcTPxI |
| WT | WT |

**RESULTS**

**Role of cTPxII in Cell Protection against Peroxide Insult**—Previous results indicate that deletion of the cTPxII gene renders cells more sensitive to peroxide insult, but the effect observed was only slight (21). Here, a very systematic study was performed by testing several peroxide concentrations, cell dilutions, and the effect of the carbon source on the protecive role of cTPxII because of the well known catabolic repression exerted by glucose (for review, see Ref. 12).

Cells were grown in synthetic medium to avoid the reaction of peroxides with extracellular components present in a rich medium such as glutathione (27). Because rich media are not so well controlled as synthetic media, reproducibility problems may occur when experiments are performed with different lots of components. Using this approach a pronounced sensitivity of the ΔcTPxII cells to TBHP treatment was found when yeast was grown in glucose or in glycerol. Of these conditions wild type cells were at least an order of magnitude more resistant to TBHP than mutant strains (Fig. 1). These results indicated that both cTPxI and cTPxII, among other antioxidants, are required to protect wild type S. cerevisiae from TBHP treatment at doses higher than 1 mM.

cTPxI and cTPxII also appeared to be very important for cell protection against TBHP treatment in glycerol as a carbon source. Under these conditions the physiology of yeast is profoundly altered since several genes are repressed by glucose (14). Again, at TBHP doses lower than 1 mM, no significant decrease in viability was observed for ΔcTPxI or ΔcTPxII cells (data not shown). However, deletion of TSA1 or TSA2 rendered yeast cells more sensitive to TBHP at higher concentrations (Fig. 1). These results indicated that both cTPxI and cTPxII were important for yeast protection against TBHP under fermentative and respiratory conditions.

A systematic investigation was also performed to find a condition where ΔcTPxII cells might show clear sensitivity to H2O2. Several conditions were tested, and no clear sensitivity of ΔcTPxII cells to H2O2 was found when yeast was grown in both glucose- and glycerol-containing media (Fig. 2). In fact, in some conditions ΔcTPxII cells appeared to be even more resistant to H2O2 than wild type strain (e.g. cells grown in glucose and treated with 1.5 mM H2O2). In contrast, a considerable effect was observed for ΔcTPxI cells exposed to H2O2 at 1.2 and 1.5 mM concentrations, especially under glucose-repressing conditions (Fig. 2). The data shown in Figs. 1 and 2 are representative of at least three independent experiments performed under the same conditions. In summary, the results presented so far indicated that both cTPxI and cTPxII are important for cell protection against TBHP.

**Analysis of ΔcTPxII Expression**—A possible explanation for the specific sensitivity of ΔcTPxII cells to organic peroxide insult could be related to the pattern of TSA2 expression, in case this gene might be induced at higher levels by organic peroxide.

**Fig. 2.** Peroxide removal activities in soluble cell extracts. The amounts of proteins in yeast cell extracts were determined by the Bradford assay (Bio-Rad protein assay kit II) using bovine serum albumin as a standard. Conditions used to measure H2O2 or organic hydroperoxide consumptions were markedly distinct and are indicated under “Results” (“Peroxide Removal Activities in Soluble Cell Extracts”).

**Catalase Assays**—Yeast cell extracts were obtained as described in the previous section. Oxygen release due to catalase activity (see Reaction 1) in cell extracts was specifically determined using Clark electrode at 30 °C (Yellow Springs Instrument Co.). The saturating oxygen concentration, which corresponds to the full scale of the electrode, was taken to be 0.225 mM (26).

Yeasts were collected and centrifuged again at 16,000 × g for 30 min to remove protein precipitates. The supernatants were collected for assay.

The remaining peroxide contents present in the supernatants were determined at different intervals by the ferrous oxidation xylenol orange assay as described previously (25). Reactions were initiated by the addition of peroxide compounds and stopped at different intervals by the addition of 200 μl HCl to the reaction mixtures. H2O2 concentration in stock solutions was checked by its absorbance at 240 nm (ε240 nm = 43.6 μM cm−1).

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**RESULTS**

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**Analysis of ΔcTPxII Expression**—A possible explanation for the specific sensitivity of ΔcTPxII cells to organic peroxide insult could be related to the pattern of TSA2 expression, in case this gene might be induced at higher levels by organic peroxide.
than by H₂O₂. Therefore, a series of Northern blot experiments were conducted to analyze TSA2 expression in wild type cells. No TSA2 transcript was detected, in any condition tested, when cells were not exposed to peroxides. However, the addition of peroxides even at very low concentrations (0.1 mM for H₂O₂ and 0.3 mM for TBHP) induced strong expression of TSA2 (Fig. 3). In contrast, cTPxI is a very abundant protein in the absence of peroxides and is only slightly induced by these oxidants (15, 18, 21). It is important to note that the probe used in our Northern blot experiments was very specific for the TSA2 gene, since no band was detected in ΔcTPxII cells exposed to 0.3 mM peroxide for 15 min (Fig. 3).

The dose-response expression pattern of TSA2 was very similar for cells exposed to H₂O₂ or TBHP. Therefore, these results could not explain the specific sensitivity of ΔcTPxII cells to organic peroxides. Consequently, TSA2 expression was also analyzed as a function of time. Maximal TSA2 expression was achieved at about 30 min of peroxide treatment followed by a sharp decrease at 60 min of treatment (Fig. 4). Again, the time dependence of TSA2 expression was very similar after treatments of cells with H₂O₂ or TBHP in both glucose- or glycerol-containing media (Fig. 4). Only the induction of TSA2 by H₂O₂ in cells grown on glucose differed slightly from the pattern described above. In this case, maximal expression was achieved at 15 min instead of 30 min (Fig. 4A).

The expression of TSA2 was also analyzed in cells grown in media containing other carbon sources and other concentrations of glucose. As described above, transcripts were only detected when cells were treated with peroxides (Fig. 5A). Finally, expression of TSA2 was measured as a function of growth phase. At later phases of growth, a low amount of transcripts could be detected in the absence of peroxides, but levels of TSA2 transcript increased greatly after peroxide treatment (Fig. 5B). The much higher induction of TSA2 by peroxide treatment than induction by glucose exhaustion is in agreement with previous reports (22, 28).

Enzymatic Properties of Recombinant cTPxII—Because specific sensitivity of ΔcTPxII cells to TBHP could not be attributed to the pattern of TSA2 induction by peroxides, the enzymatic properties of cTPxII were also analyzed. We hypothesized that cTPxII might be much more efficient in the removal of organic peroxides than in decomposition of H₂O₂. It was reported before that the enzymatic activity of cTPxII is higher for H₂O₂ than for TBHP using an assay based on NADPH oxidation (2). In this case only one concentration of peroxide was analyzed (1 mM). Although the enzymatic parameters of other yeast peroxiredoxins are known (29–31), Kₘ and Kₗ for cTPxII and Kₗ for cTPxI have never been established before. Therefore, we analyzed the properties of cTPxII in detail and determined the enzymatic parameters. Additionally, the enzymatic data of cTPxI and cTPxII were compared using the same thioredoxin and thioredoxin reductase preparations.

cTPxII was equally efficient in the removals of H₂O₂ and of TBHP (Fig. 6), and all the three Kₘ/Kₗ ratios for cTPxII were in the same order of magnitude (10⁴ M⁻¹ s⁻¹) (Table I). As described before (2), cTPxI was more efficient than cTPxII in the removal of H₂O₂ (Fig. 6A and Table I); however, the catalysis of TBHP decomposition by cTPxI and cTPxII occurred with about the same efficiency (Fig. 6B). Because cTPxII decomposed all the peroxides with approximately the same efficiency (Table I), it does not appear that the enzymatic properties of cTPxI or cTPxII can be implied with the specific sensitivity of ΔcTPxII cells to organic peroxides.

Peroxide Removal Activities in Soluble Cell Extracts—One alternative explanation for the specific sensitivity of ΔcTPxII cells to organic peroxides might be related to the role of other antioxidant enzymes than peroxiredoxins. For instance, catalase and cytochrome c peroxidase I are heme-dependent proteins that exclusively decompose H₂O₂ through the catalysis of the Reactions 1 (see the Introduction) and 3, respectively.

2 Cytochrome c (Fe²⁺) + H₂O₂→2 cytochrome c (Fe³⁺) + H₂O

REACTION 3

Therefore, it would be expected that the repertoire of antioxidant enzymes available to reduce H₂O₂ would be higher than that available to decompose organic peroxides. Accordingly, the ability of soluble cell extracts to remove H₂O₂ was
much higher than their ability to degrade TBHP. The yeast cell extracts whose total protein concentration was equal to 0.25 g/l decomposed a large amount of H2O2 added (Fig. 7A), whereas no removal of TBHP was detected under these conditions (data not shown). It is noteworthy to observe that almost all of the H2O2 consumption was inhibited by azide (data not shown), indicating that heme-dependent proteins, most probably catalase, mediated this process (see "Discussion"). The total H2O2 removal activities of all yeast cell extracts were in the same order of magnitude, although cell extracts from ΔcTPxII cells have significantly higher capacity to decompose this oxidant (Fig. 7A).

**Fig. 3. Effect of peroxide dose on TSA2 expression.** Northern blot analysis of RNA isolated from mid-logarithmic phase culture (A600 nm = 0.8). All of samples were treated with peroxide for 15 min. Panels A and B refer to H2O2 treatment, whereas panels C and D refer to TBHP treatment. Panels A and C refer to glucose as the carbon source, whereas panels B and D refer to glycerol as the carbon source. This figure is representative of at least three similar experiments.

**Fig. 4. Kinetics of TSA2 induction by hydroperoxides.** Northern blot analysis of RNA isolated from mid-logarithmic phase cultures (A600 nm = 0.8). Time 0 corresponds to untreated cells. Panels A and B refer to H2O2 (0.3 mM) treatment, whereas panels C and D refer to TBHP (0.5 mM) treatment. Panels A and C refer to glucose as the carbon source, whereas panels B and D refer to glycerol as the carbon source. This figure is representative of at least three similar experiments.
Fig. 5. **TSA2 expression under fermentative and respiratory conditions**. A, effect of carbon source on TSA2 expression. Northern blot analysis of TSA2 from cells grown in media containing different carbon sources, which are described at the bottom of the figure. RNA was isolated from mid-logarithmic phase cultures (A600 nm = 0.8). Where indicated, TBHP was added at 0.5 mM concentration during different growth phases. Northern blot analysis of RNA isolated from wild type cells. The growth phases are indicated at the bottom of the figure. Treated (0.5 mM of TBHP) and untreated samples are indicated by + or − signals, respectively. The carbon source used in all samples was glucose. This figure is representative of at least three similar experiments.

Next, the catalase activities were specifically measured by oxygen release (see Reaction 1). Catalase activity in ΔcTPxII cells was about 2-fold higher than the catalase observed in wild type and ΔcTPxI cell extracts (Fig. 7B). As expected, oxygen release in all yeast cell extracts was fully inhibited by azide (data not shown).

Finally, the concentration of yeast cell extracts and the interval for the analysis of peroxide consumption were increased to detect TBHP removal. After 2 h, cell extracts (4 μg/μl) from ΔcTPxII cells decomposed less TBHP than wild type cells (116 μM ± 3.53 for ΔcTPxII and 139 μM ± 12.72 for wild type cells). These differences between ΔcTPxII and wild type cells were statistically significant for p values =0.05 (determined from paired t tests). Therefore, ΔcTPxII cells presented the highest H2O2 removal activity and the lowest TBHP removal ability, which should be related to their response to these peroxides.

In summary, our results indicated that heme-dependent proteins, most probably catalase, in ΔcTPxII cells are responsible at least in part for the resistance of this strain to H2O2 (Fig. 2). Because heme-dependent peroxidases are specific for H2O2 (6), they could not cooperate with peroxiredoxins in the protection of ΔcTPxII cells to TBHP treatment. Therefore, the unique phenotype of ΔcTPxII cells (high sensitivity to TBHP and resistance to H2O2) is probably related to the induction of catalase activity in this strain.

**Discussion**

In this work we report an extensive analysis of the viability of yeast strains with deletions in the genes encoding cTPxI and cTPxII. Interestingly, ΔcTPxII cells were specifically sensitive to TBHP and resistant to H2O2 insult, whereas ΔcTPxI strain was more sensitive than wild type strain to both kinds of peroxides (Figs. 1 and 2). Some hypotheses were tested to explain this phenotype of ΔcTPxII cells: 1) TBHP induces higher levels of cTPxII than H2O2, 2) cTPxII is more efficient in the enzymatic removal of organic peroxides than in the enzymatic removal of H2O2, and 3) there is alteration of the levels of antioxidants other than cTPxI and cTPxII.

Because the patterns of TSA2 induction by H2O2 and by TBHP were very similar (Figs. 3 and 4), hypothesis 1 could be ruled out. Enzymatic assays with recombinant peroxiredoxins were conducted to test hypothesis 2. In our hands, cTPxII catalyzed the reduction of both organic hydroperoxides and H2O2 with almost the same efficiency (10^4 M^-1 s^-1, Table I), indicating that hypothesis 2 should be rejected. As a matter of comparison, other thiol-dependent peroxidases from S. cerevisiae, namely cTPxIII (also known as Ahp1, type II TPx, or TSA II), is an order of magnitude more efficient in the removal of organic peroxides than in the removal of H2O2 (29).

cTPxI decomposed H2O2 more efficiently than cTPxII, although in both reactions, catalysis was of the same order of magnitude (Fig. 6A, Table I). The catalysis of organic peroxides by these two proteins was also in the same range. These results indicate that cTPxI and cTPxII behave similarly, supporting the observation that these two proteins decompose peroxides at comparable levels in yeast cells (21). These results were expected since cTPxI and cTPxII share 86% of identity (96% of similarity) in their amino acid sequence. However, in a previous report (2), it was shown that cTPxI had the lowest TBHP removal activity among the peroxiredoxines studied, which is in contrast with our results showing that cTPxI is only slightly more efficient than cTPxII in the removal of organic peroxides (Fig. 6B, Table I). This discrepancy is perhaps explained by the fact that only one peroxide concentration (1 mM) was tested in the experiments described by Park et al. (2). Because it is well...
known that peroxides at high concentrations inhibit peroxiredoxins (32), perhaps cTPxII is inhibited to a higher extend than cTPxI. In any case it is important to emphasize that the kinetic parameters measured here are in the same range of the values obtained for others peroxiredoxins belonging to the same 2-cys peroxiredoxin category (32).

Analyses of gene deletions are complex because several processes can be affected in null mutants besides the absence of the respective protein. As an example, deletion of TSA1 gene promotes the induction of several other genes related to the glutathione system (4). Based on that, our alternative hypothesis for the unique phenotype ΔcTPxII strain (hypothesis 3) seems to explain the results described herein. For instance, heme-dependent proteins, such as catalase and cytochrome c peroxidase, specifically catalyze the decomposition of H2O2 but not of organic peroxides (for review, see Ref. 6).

In wild type cells, cTPxI is a very abundant protein and, therefore, at low peroxide concentrations it could cope with oxidative stress, especially in cells in the log phase (15, 19). At high peroxide concentrations, other enzymes may be necessary to protect yeast. In the case of high levels of organic peroxides, it appears that cTPxII and cTPxI (among other antioxidants) would be responsible for the antioxidant protection. In the case of the oxidative stress insult by high levels of H2O2, most likely catalase and cytochrome c peroxidases cooperate with cTPxI and cTPxII.

To test hypothesis 3, peroxide removal activities were determined in several conditions and in several cell extracts. Our results indicated that the capacity of yeast to decompose H2O2 is much higher than the removal of TBHP. After 10 min of incubation, no removal of organic peroxide by cell extracts was detected (data not shown), whereas about 40–66% of added H2O2 was decomposed within same period of incubation (Fig. 7A). Very importantly, almost 100% of H2O2 decomposition

**Table I**

Enzymatic Parameters for peroxiredoxins

| Peroxide | cTPxI | cTPxII | cTPxI | cTPxII |
|----------|-------|--------|-------|--------|
| Peroxide | Vmax  | K<sub>cat</sub> | K<sub>cat</sub>/K<sub>1m</sub> | Vmax  | K<sub>cat</sub> | K<sub>cat</sub>/K<sub>1m</sub> |
| H<sub>2</sub>O<sub>2</sub> | 0.66  | 12.0   | 0.31  | 2.6 × 10<sup>4</sup> | 0.39  | 13.8   | 0.18  | 1.3 × 10<sup>4</sup> |
| TBHP    | 0.61  | 7.9    | 0.29  | 3.6 × 10<sup>4</sup> | 0.29  | 5.1    | 0.14  | 2.7 × 10<sup>4</sup> |
| CHP     | 0.56  | 17.1   | 0.26  | 1.5 × 10<sup>4</sup> | 0.28  | 4.5    | 0.14  | 3.0 × 10<sup>4</sup> |

**Figure 6.** NADPH oxidation catalyzed by cTPxI and cTPxII. NADPH oxidation was monitored as A<sub>340 nm</sub> in a 1-ml reaction mixture containing 50 mM Hepes-NaOH, pH 7.4, 100 μM DTPA, 1 mM azide, 0.225 μM Trx2, 0.075 μM Trx1, 2.1 μM cTPxII, 0.18 mM NADPH, and 20 μM peroxide (A = H<sub>2</sub>O<sub>2</sub> and B = TBHP). Reactions were incubated at 30 °C and were initiated by peroxide addition. This figure is representative of at least three similar experiments (dotted line, without peroxiredoxin; □, cTPxI; ×, cTPxII).
observed in Fig. 7 was inhibited by azide (data not shown), indicating an important role of heme-dependent proteins, most probably catalase. Accordingly, it has been shown that H2O2 decomposition, in the absence of reductants, was fully abolished in an acatalasemic yeast strain (33). In fact, catalase activity was specifically detected in all yeast cell extracts due to its ability to release oxygen (Fig. 7B). Both H2O2 removal and catalase activity were significantly higher in ΔcTPxI wild type (but not between ΔcTPxI and wild type) were statistically significant for p values ≤ 0.05 (determined from paired t tests) and are indicated with the asterisk. WT, wild type.

In both ΔcTPxI and ΔcTPxII cells, there are catalase and cytochrome c peroxidase available to decompose H2O2, but only the first strain is more sensitive than wild type cells to this oxidant (Fig. 2). Several factors may explain this phenomenon; (i) cTPxI is much more abundant than cTPxII in basal conditions; (ii) catalase activity determined in ΔcTPxII cells was twice the activity found in ΔcTPxI strain (Fig. 7), (iii) cTPxI was more efficient than cTPxII in the removal of H2O2 (Table I, Fig. 6A, and Ref. 2), and (iv) transcription of TSA2 gene is induced in ΔcTPxI cells, but TSA1 mRNA levels are not increased in ΔcTPxII cells (21).

TBHP removal by soluble cell extracts is also in agreement with the hypothesis 3. To measure TBHP consumption the concentration of cell extracts were increased 16 times in relation to the experiments of H2O2 consumption. This is probably related with the fact that both wild type and mutant cells cannot account with catalase or cytochrome c peroxidase to decompose organic peroxides because these enzymes are specific for H2O2 (for review, see Ref. 6). Thus, the repertoire of antioxidant enzymes available to decompose organic peroxides in S. cerevisiae is limited to fewer enzymes. Consequently, this microorganism might be more dependent on cTPxI and cTPxII to protect itself from this oxidant. In fact, deletion of TSA2 gene rendered yeast more sensitive to TBHP (Fig. 1) and reduced the capacity of this microorganism to decompose this oxidant.

The mechanisms involved in cell susceptibility to peroxides appeared to be distinct in the ΔcTPxI and ΔcTPxII mutants. In the case of ΔcTPxII cells, a significant up-regulation of catalases was observed (Fig. 7), which should contribute for the resistance of this strain to H2O2 (Fig. 2). In some cases (e.g., cells grown in glucose-containing media and exposed to 1.5 mM H2O2), ΔcTPxII strain was even more resistant than wild type cells. On the other hand, this strain presented high TBHP sensitivity (Fig. 1). The factors involved in this phenotype are not evident, but they should not solely depend on the absence of cTPxII proteins because this peroxidase is not an abundant protein in basal conditions (22). Probably, proteins other than cTPxI and cTPxII are involved in this phenomenon. One of these proteins could be the recently described mitochondrial glutathione reductase, whose activity is low in ΔcTPxII mitochondria (35, 36). Consequently, the low levels of reduced glutathione in the mitochondria of ΔcTPxII cells should limit the supply of reducing equivalents to phospholipid glutathione peroxidase enzymes and, therefore, impair the decomposition of organic peroxides by this pathway (3, 4). Along with cTPxI, cTPxII, and phospholipid glutathione peroxidase proteins, cTPxIII is another important protein in the protection of S. cerevisiae against TBHP (29, 37). ΔcTPxI cells are very sensitive to both kinds of peroxides probably because they do not possess cTPxI (a very abundant protein), one of the most important proteins of this microorganism.
defense against these oxidants (2, 4, 5, 7, 15, 18–21, 24). The signaling network involved in the response of yeast to the different kinds of peroxides is very complex. In the case of mTPxI (also known as Prx1p), at least two different pathways are involved in the regulation of its expression, one dependent on heme and the other on CAMP as signaling agents (16). The zinc-finger transcriptional regulator MsnP/Msn4p regulates the induction of mTPxI by carbon starvation, whereas another transcriptional regulator, Hap1p, is involved in the induction of mTPxI by H$_2$O$_2$ (16). Recently, it was reported that both MsnP/Msn4p and Hap1p also regulate cTPxII expression among other transcriptional regulators (22, 28). These results together with microarray data (38) indicate that expression of mTPxI and cTPxII may share at least part of the same regulatory pathways. Like mTPxI, cTPxII is highly inducible by peroxides and that defense are not completely understood, the results presented in this report clearly show that cTPxI and cTPxII are key components of yeast defense against organic hydroperoxides and that catalases and peroxiredoxins cooperate with them in the protection of cells against H$_2$O$_2$ insult. Our results together with data from the literature indicate that, although all the peroxiredoxin isoforms share the same enzymatic activity (thiol-dependent peroxidase), they are not totally redundant proteins. Because yeast has proved to be a good model to higher eukaryotes, this assumption may also be valid for the peroxide detoxification systems of these organisms.

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