Cooperation of Yeast Peroxiredoxins Tsa1p and Tsa2p in the Cellular Defense against Oxidative and Nitrosative Stress*

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Peroxiredoxins are a family of antioxidant enzymes conserved from bacteria to humans. In Saccharomyces cerevisiae, there exist five peroxiredoxins, among which Tsa2p shares striking homology with the well described Tsa1p but has not been extensively studied. Here we report on the functional characterization of yeast tsa2Δ mutants and the comparison of TSA1 with TSA2. The tsa2Δ and tsa1Δ tsa2Δ cells grew normally under aerobic conditions. However, the tsa1Δ tsa2Δ mutant yeast was more susceptible to oxidants than either tsa1Δ or tsa2Δ cells. Notably, the tsa1Δ tsa2Δ yeast was also hypersensitive to peroxynitrite and sodium nitroprusside. This phenotype was rescued by the expression of either the TSA1 or TSA2 gene. The demonstration of a peroxynitrite reductase activity of Tsa2p in vitro points to a pivotal role for peroxiredoxins in the protection against nitrosative stress. In yeast cells, Tsa1p and Tsa2p exhibited comparable antioxidant activity. While the basal expression level of TSA1 was significantly higher than that of TSA2, the transcription of TSA2 was stimulated more potently by various oxidants. In addition, TSA2 was activated in tsa1Δ cells in a Yap1p-dependent manner. Taken together, our findings implicate the cooperation of Tsa1p and Tsa2p in the cellular defense against reactive oxygen and nitrogen species.

Living organisms are constantly exposed to reactive oxygen species (ROS)† that are produced during metabolism or in response to external stimuli (1). In addition to ROS, reactive nitrogen species (RNS) have emerged as another source of oxidative and nitrosative stress (2, 3). Both ROS and RNS have been implicated in various physiological and pathological processes including metabolism, immunity, inflammation, cell signaling, transcriptional regulation, and apoptosis (1–3). The cellular defense against oxidative and nitrosative stress is important for homeostasis and survival.

Antioxidant enzymes are important components of the cellular defense system against ROS and RNS. In addition to well documented antioxidant enzymes such as superoxide dismutase and catalase, a novel family of peroxidases, designated peroxiredoxins, has recently been characterized (4–6). Peroxiredoxins are found in all organisms ranging from bacteria to humans. They are thought to be active peroxidases supported by thioredoxin and other electron donors (5, 7). The amino acid sequences around the peroxidatic center characterized by a cysteine residue are highly conserved. The oxidation of the cysteine induced the formation of a decameric structure comprising five dimers (8, 9). In a more recent study, bacterial peroxiredoxin AhpC has been shown to be peroxynitrite reductase (10), thus conferring resistance to RNS (11). It remains to be seen whether eukaryotic peroxiredoxins can generally scavenge peroxynitrite in addition to hydrogen peroxide and directly protect cells from RNS.

Multiple subtypes of peroxiredoxins are often found in one species (6). Thus, there are five peroxiredoxin genes (TSA1/cPrx1/YML026W, TSA2/cPrxII/YDR453C, BCP/nTPx/DOT5/YIL101W, 1CPrx/mTPx/YBL064C, and PMP20/AHP1/cPrxIII/YLR109W) in Saccharomyces cerevisiae (5, 12–16). Tsa1p is the first identified peroxiredoxin, and it has been shown to be the major thiolreactive peroxidase in the cytoplasm (5, 16). 1CPrx localizes to mitochondria (15), and PMP20 resides in peroxisomes (12–14). Different peroxiredoxins in yeast are thought to have redundant and nonredundant functions (16). Notably, four of the five yeast peroxiredoxins have mammalian orthologs (4, 5, 17–28). Thus, the budding yeast represents an attractive model for the study of peroxiredoxins.

To date, only a limited number of enzymes in the large family of peroxiredoxins have been characterized for function. Fundamental questions as to whether and how peroxiredoxins scavenge RNS and ROS remain unanswered. Coordinated efforts using different biological systems are necessary for functional studies. One route toward understanding the physiology of peroxiredoxins is through the phenotype of null mutants in yeasts. Among the yeast peroxiredoxins, Tsa2p is highly homologous to the well described Tsa1p (5, 7). However, Tsa2p has been shown to be very different from Tsa1p and other peroxiredoxins in yeast. Surprisingly, TSA2-null mutants suffered a severe growth retardation characterized by the accumulation of G1 cells and were insensitive to oxidants (16). To shed additional light on the physiological functions of Tsa2p, we constructed and characterized tsa2Δ and tsa1Δ tsa2Δ mutant yeasts. We also compared the function and regulation of TSA1 and TSA2. The tsa2Δ and tsa1Δ tsa2Δ cells were indistinguishable from wild type under aerobic conditions. However, the tsa1Δ tsa2Δ mutant yeast grew more slowly in the presence of ROS and RNS. The antioxidant properties of Tsa1p and Tsa2p were comparable. The basal expression level of TSA1 was significantly higher, but the transcription of TSA2 was more potently activated in response to ROS or RNS and to the loss of Tsa1p protein. We also provide the first evidence for the

* This work was supported by a grant from the Hong Kong Research Grants Council (Project HKU 7240/06M) to D.-Y. J. and a research initiation grant from the Committee on Research and Conference Grants, University of Hong Kong (to D.-Y. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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† The abbreviations used are: ROS, reactive oxygen species; RNS, reactive nitrogen species; DCF, 2′,7′-dichlorodihydrofluorescein; PBS, phosphate-buffered saline; SNP, sodium nitroprusside; t-BHP, t-butylhydroperoxide; DTT, dithiothreitol; RT, reverse transcription.

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peroxynitrite reductase activity of Tsa2p. Our findings suggest that Tsa2p cooperated with Tsa1p to protect the yeast cells from oxidative and nitrosative stress.

EXPERIMENTAL PROCEDURES

Strains and Media—The S. cerevisiae strain BY4741 and its isogenic strains (Table I) (29) were used in this study. Yeasts were grown in YPD (2% yeast extract, 1% peptone, and 2% dextrose in H2O), YPG (2% yeast extract, 0.5% peptone, and 2% glucose in H2O), or synthetic dextrose (SD) medium lacking leucine (SD–Leu), histidine (SD–His), or uracil (SD–Ura). Cell transformation was performed using the lithium acetate method (26) or the lithium acetate–polyethylene glycol method (27). Other chemicals were from Sigma.

Construction of tsa1 and tsa2 

Construction of TSA1 and TSA2 Expression Plasmids—The open reading frame was excised with lacZ

Construction of tsa1 and tsa2 Mutants—DNA fragments containing TSA1 or TSA2 open reading frame were PCR-amplified from S. cerevisiae genomic DNA (Novagen). The oligonucleotide primers are 5′-CGGATCCGGTACGCGCAAT (forward) and 5′-CCGTCGACGGAUCCGCGCTC (reverse) (for TSA1) (expected product size: 869 bp) and 5′-CGGATCCGGTGACGCTCTCTCTCT (forward) and 5′-CCGTCGACGTTCATCATATGCCAT (reverse) (for TSA2) (product size: 906 bp), respectively. These primers introduce HincII and XhoI (reverse) sites (underlined). The resulting PCR fragments were gel-purified, digested with EcoRI and XhoI, and cloned into plasmid pBlueScript II SK (Stratagene). The +52 to +447 nucleotides from the TSA1 open reading frame were excised with Sall and HindIII and replaced with the HIS3 gene from pDG201 (a gift from L. Deren). The +54 to +452 sequences from the TSA2 open reading frame were removed with HindIII and NotI and substituted with the LEU2 gene from plasmid pGAD424 (CLONTECH). The tsa1 disruption strains were obtained by allele replacement using a one-step displacement method (30). The DNA fragment, which contains the HIS3 selectable marker flanked by the TSA1 sequence, was transformed into different strains. The tsa2 disruption strains were obtained by the same approach, except that the DNA fragment containing the LEU2 selectable marker flanked by the TSA2 sequence was used. The tsa1::HIS3 and tsa2::LEU2 genotypes were verified by PCR and Southern analysis.

Construction of tsa1 and tsa2 Expression Plasmids—To construct inducible expression vectors for TSA1 and TSA2, DNA fragments harboring TSA1 and TSA2 were amplified from yeast genomic DNA using primers 5′-TGTGACACTCTGCAAG (forward) and 5′-GGATGTTGAGTGA (reverse) (for TSA1) (expected size of product: 264 bp). The TSA1-specific primers are 5′-CTTGACACATACAGAAG and 5′-CAAGAATGGTTTG (expected size of product: 247 bp). The TSA2-specific primers are 5′-ACTGGAAAGTATATAA and 5′-AATAAGGATTACA (expected size of product: 423 bp). These sequences were used to clone TSA1 and TSA2 fragments containing the promoter and the first few codons of the open reading frame were amplified from S. cerevisiae genomic DNA (Novagen) using Fpy DNA polymerase (Invitrogen). The oligonucleotide primers are 5′-CCGGAACGCTGTAACAGCACCGCAAC (forward) and 5′-CCGACATTACGACGTGTTTTTCT (reverse) (for TSA1) and 5′-CGGATCCGGCAAGCCACAGCTC (forward) and 5′-CCGAAATTCCTACGGCGTCTTC (reverse) (for TSA2), respectively. The restriction sites for subcloning are underlined. The fragments were cloned first into pBlueScript II SK and then inserted into the lacZ plasmid pEB39 (a gift from E. A. Elion). For insertion of the lacZ gene into the chromosomal TSA1 and TSA2 loci, the TSA1-lacZ and TSA2-lacZ plasmids were digested with HindIII and ClaI. The DNA fragments harboring the TSA1 and TSA2 genes were ligated into plasmid pGAD424 and the resulting plasmids were digested by MuniII and BgII and integrated into the yeast genomic DNA by homologous recombination. In the TSA1-lacZ mutant yeast, the lacZ gene was driven by the authentic TSA1 promoter, and the TSA1 gene was under the control of another copy of the previously cloned TSA1 promoter (−1 kb). Likewise, in the TSA2-lacZ yeast, the lacZ gene was driven by the authentic TSA2 promoter, and the TSA2 gene was under the control of another copy of the previously cloned TSA2 promoter (−1 kb). The sequences of all PCR products were confirmed by DNA sequencing, and no mutation had been introduced.

ROS Detection—Intracellular redox levels were measured by fluorescence microscopy or by flow cytometry using the fluorescent dye 2′,7′-dichlorofluorescein diacetate. Cells were grown in YPD medium until A590 reached 0.5. Hydrogen peroxide was added to a final concentration of 10 μM. After an additional incubation for 15 min, cells were collected by centrifugation from 2 ml of culture and then washed three times with phosphate-buffered saline (PBS). Cells were resuspended in PBS with 10 μM 2′,7′-dichlorofluorescein diacetate (Molecular Probes, Inc., Eugene, OR) and incubated at 28 °C for 1 h. The dye can react specifically with hydrogen peroxide to give a highly fluorescent 2′,7′-dichlorofluorescein diacetate. Cells were washed three times with PBS, mounted onto slides, and examined under a confocal fluorescence microscope (Zeiss). An argon ion laser with an emission line at 488 nm was used to excite DCF. Alternatively, the cells were disrupted by glass beads, and the supernatant was collected by centrifugation from 2 ml of culture and then washed three times with PBS.

Flow Cytometric Analysis of DNA Content—Yeast were grown in YPD medium until A590 reached 0.5 and exposed to oxidants for 0.5 h. Cells were then diluted and plated for colony survival.

β-Galactosidase Assay—β-Galactosidase levels were measured with treated or untreated mid-log phase cells as previously described (32, 33). α-Nitrophenyl-β-D-galactopyranoside was used as substrate activities. Activities were given in A420 units/min/mg of protein.

Flow Cytometric Analysis of DNA Content—After two washes with water, cells were fixed with 70% ethanol for 12 h at 4 °C and then treated with RNase A (1 mg/ml) in PBS for 30 min at 37 °C. Then proteinase K (10 mg/ml) was added, and the cells were further incubated for 1 h at 55 °C. Cells were stained with propidium iodide (50 μg/ml) in 10 mM Tris-HCl (pH 8.0), 10 mM NaCl. Stained cells were subsequently diluted in PBS, and for each sample the DNA content in 10,000 cells was determined with a FACScan flow cytometer. Flow cytometric analysis was performed with EXPQ program (EPICS).

Expression and Purification of Histidine-tagged Tsa2p—A DNA fragment containing TSA2 gene was PCR-amplified from E. coli BL21(DE3) (Novagen) and cloned into expression vectors pET-28a (Novagen). Histidine-tagged Tsa2p was expressed in E. coli BL21 (DE3) and purified using procedures recommended by Novagen. The purified His-Tsa2p was reduced by 5 mM tris(2-carboxyethyl)phosphine (TCEP) in a buffer containing 10 μM 2′,7′-dichlorofluorescein diacetate and then exposed to oxidants for 0.5 h. Cells were then disrupted by glass beads, and the supernatant was collected by centrifugation from 2 ml of culture and then washed three times with PBS.

Peroxynitrite Reductase Assay—The peroxynitrite-mediated oxidation of dihydrorhodamine 123 to rhodamine was followed as previously

3586

Yeast Peroxiredoxins Tsa1p and Tsa2p in Antioxidant Defense

Table I

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<tr>
<th>Strain</th>
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RESULTS

Molecular Evolution of Yeast Tsa1p and Tsa2p—The yeast *S. cerevisiae* is a useful model for studies of eukaryotic antioxidant enzymes and redox signaling (1). Budding yeast Tsa1p is the first identified peroxiredoxin (5, 34) and is one of the best-studied members in this family (5, 7, 16, 34–36). Among the five peroxiredoxins in yeast, Tsa2p is unique in having striking homology with Tsa1p. Notably, 86% of the amino acid residues in Tsa1p and Tsa2p are identical, and 96% are similar. The separation of Tsa2 with TSA1 probably occurred after the speciation of budding yeast, as the result of a gene duplication event. The high degree of sequence homology suggests a conservation of function. Studies of yeast Tsa1p and Tsa2p may derive novel insights into the biology of closely related peroxiredoxins in other species.

Growth Phenotypes of the Δtsa2 Strains—The five mutants deleted for individual peroxiredoxins have been shown to be viable (16, 35). This raises two not mutually exclusive possibilities that may explain the phenotype. First, there could be functional overlap between different peroxiredoxins. Second, all peroxiredoxins might be dispensable for viability because of functional overlap between peroxiredoxins and other antioxidant enzymes. One way to explore these possibilities is through the construction of multiple peroxiredoxin-null mutants.

We also noted that the TSA2-null strain exhibited a unique phenotype characterized by severe growth retardation with the accumulation of G1 cells (16). In addition, unlike other peroxiredoxin-null mutants, the yeast strain deleted for TSA2 was insensitive to oxidant challenge (16). A closer examination of the tsa2Δ phenotype is required to further elucidate the underlying mechanisms.

To address the above issues and to characterize the cellular functions of yeast Tsa1p and Tsa2p, we constructed tsa2Δ and tsa1Δ tsa2Δ strains by replacing an internal fragment within the coding region of TSA2 with the LEU2 marker. PCR (Fig. 1A) and Southern blotting (Fig. 1B) were performed to confirm the insertion of LEU2 into the TSA2 gene. In addition, no TSA2 mRNA was detected in tsa2Δ or tsa1Δ tsa2Δ cells by Northern blotting (Fig. 1C). All three experiments consistently demonstrated the disruption of the TSA2 gene in the tsa2Δ and tsa1Δ tsa2Δ strains.

The tsa1Δ, tsa2Δ, and tsa1Δ tsa2Δ strains were all viable, indicating that neither TSA1 nor TSA2 is essential for normal aerobic growth. In addition, both the tsa2Δ and the tsa1Δ tsa2Δ strains showed wild-type growth rate in rich (YPD; Fig. 2A) or minimal (SD; data not shown) medium. The tsa2Δ and tsa1Δ tsa2Δ mutants were indistinguishable from the isogenic wild-type BY4741 strain either in growth (Fig. 2A) or in cell morphology (data not shown). Next, we performed flow cytometric analysis to assess the DNA content and to compare the cell cycle profiles of the wild-type and mutant yeasts (Fig. 2B). Again, the tsa2Δ (panel 3) and tsa1Δ tsa2Δ (panel 4) strains did not show any difference from the BY4741 (panel 1) or tsa1Δ (panel 2) cells. Notably, the distribution profiles of G1/S and G2/M cells in the four strains were very similar. In sharp contrast to a previous report (16), the G1 peak in either the tsa2Δ yeast or the tsa1Δ tsa2Δ double mutant is not higher than in the BY4741 or tsa1Δ strain (i.e. we did not observe the accumulation of G1 cells in the tsa2Δ strains).

Sensitivity of tsa2Δ Strains to Oxidants—The exponentially growing TSA2-null yeast cells have been shown to be insensitive to oxidants (16). Because the behaviors of our tsa2Δ strains were different from the reported slow growth phenotype, we sought to reexamine the sensitivity of our tsa2Δ strains to oxidants and the antioxidant response in these strains.

First we challenged the four yeast strains (wild-type BY4741, tsa1Δ, tsa2Δ, and tsa1Δ tsa2Δ) with H2O2 and t-butylhydroperoxide (t-BHP). The sensitivity was assessed in the spot assay (Fig. 3A). Consistent with our findings from growth rate studies (Fig. 2A) and flow cytometric analysis (Fig. 2B), the four strains grew normally in the absence of oxidant insult (Fig. 3A, lanes 1–3). However, the tsa2Δ cells were more sensitive to peroxides than the wild type BY4741 but less sensitive than the tsa1Δ cells (Fig. 3A, lanes 6 and 9). This implicates that Tsa2p plays a significant, albeit secondary, role in the cellular response to oxidants. Among the four isogenic strains, the double mutant tsa1Δ tsa2Δ is most sensitive to oxidant challenge, suggesting that Tsa1p and Tsa2p cooperate in the antioxidant defense.

The antioxidant properties of peroxiredoxins to protect cells from oxidant insult are ascribed to their ability to scavenge hydrogen peroxide (7). To follow more closely the removal of H2O2, we measured the levels of intracellular H2O2 in the four strains by confocal fluorescence microscopy using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (Fig. 3B). This dye has been widely used for ROS detection (17), and it reacts specifically with H2O2 to produce a highly fluorescent DCF. We observed that the DCF fluorescence in all three mutants (Fig. 3B, panels 4, 6, and 8) significantly increased compared with that in the parental wild-type strain BY4741 (panel 2). The relative intensity of the fluorescence in the four strains was in the following order: tsa1Δ tsa2Δ > tsa1Δ > tsa2Δ > BY4741 (Fig. 3B). This order is generally consistent with the sequence of sensitivity to oxidants (Fig. 3A).

Sensitivity of tsa1Δ and tsa2Δ Strains to RNS—Bacterial peroxiredoxin AhpC has peroxynitrite reductase activity (10) and therefore confers resistance to RNS (11). Yeast Tsa1p and Tsa2p are in the same subfamily as AhpC. However, it remains to be seen whether Tsa1p, Tsa2p, and eukaryotic peroxiredoxins can protect cells from RNS. To address this question, we
tested the sensitivity of TSA1- and/or TSA2-null strains to peroxynitrite and sodium nitroprusside (SNP).

As a product of nitric oxide and superoxide, peroxynitrite (ONOO\(^-\)) is a potent oxidizing and nitrating species with mutagenic, proapoptotic, and cytotoxic activities. Peroxynitrite has been implicated in the pathogenesis of various diseases (37). SNP is a nitric oxide donor frequently used in biomedical research and in clinical practice (37). When the four yeast strains were exposed to 1 mM peroxynitrite, the \( \text{tsa1}^\Delta \), \( \text{tsa2}^\Delta \), and \( \text{tsa1}^\Delta \text{tsa2}^\Delta \) strains were significantly more susceptible than the wild-type BY4741 (Fig. 4A). Similarly, the \( \text{tsa1}^\Delta \) yeast was more sensitive to 2 mM SNP than BY4741. While no difference in the sensitivity of the \( \text{tsa2}^\Delta \) and the parental BY4741 strains to SNP was noted, the \( \text{tsa1}^\Delta \text{tsa2}^\Delta \) double mutant displayed an increased sensitivity compared with the BY4741 and \( \text{tsa1}^\Delta \) strains (Fig. 4B). Collectively, these results support a model in which Tsa1p cooperates with Tsa2p in the protection against RNS.
To verify the specificity of action, we performed complementation assays with TSA1 or TSA2. A centromere expression plasmid for TSA1 was transformed into the tsa1Δ, tsa2Δ, and tsa1Δ tsa2Δ strains (Fig. 4C). The growth rates of the four strains were indistinguishable, indicating that the expression of TSA1 driven by the TSA1 promoter fully complemented the deficiency of TSA1 and/or TSA2. In contrast, the expression of TSA2 driven by the TSA2 promoter complemented the defects in TSA1 and/or TSA2 partially (Fig. 4D). Conceivably, the incompleteness of the effect may arise from the inefficient expression and/or the lower antioxidant activity of the protein. To shed additional light on this, we swapped the TSA1 and TSA2 promoters and constructed plasmids pTSA1-2 and pTSA2-1. The expression of Tsa2p from plasmid pTSA1-2 was regulated by the TSA1 promoter. Vice versa, the TSA2 promoter was used to drive the expression of Tsa1p from pTSA2-1. Interestingly, the tsa1Δ tsa2Δ strain harboring the pTSA2-1 plasmid (Fig. 4E, curve 5) was as resistant to SNP as the BY4741 yeast (Fig. 4A, curve 1), indicating that the expression of Tsa2p alone sufficiently protected cells from SNP challenge. Likewise, the pTSA2-1 plasmid conferred substantial but not full protection against SNP (Fig. 4E, curve 6). One interpretation for the partial effect is that the TSA2 promoter is less potent than the TSA1 promoter. This hypothesis is supported further by the facts that both TSA1 (Fig. 4C) and TSA1–2 (Fig. 4E, curve 5) are fully competent in the defense against SNP.

One previous study suggests that the specific thioredoxin-dependent peroxidase activity of Tsa1p in vitro is probably reflected by the formation of an interchain disulfide bond, and the appearance of dimeric Tsa2p on gel filtration. When this preparation of reduced His-Tsa2p was added to the reaction containing peroxynitrite and dihydrorhodamine 123, we observed a pronounced inhibition of rhodamine 123 formation (Fig. 5C, lanes 1 and 2). Moreover, we demonstrated that the oxidation of His-Tsa2p by peroxynitrite (Fig. 5, lanes 1 and 2) can be reversed by the addition of β-mercaptoethanol (lane 2). Because the appearance of dimeric Tsa2p probably reflects the formation of an interchain disulfide bond, peroxynitrite may oxidize Tsa2p reversibly on cysteine residues. Thus, Tsa2p acts as a peroxynitrite reductase in vitro.

Comparison of the H2O2-scavenging Activities of Tsa1p and Tsa2p in Vivo—In light of the ability of Tsa1p and Tsa2p to protect cells against RNS (Fig. 4), we asked whether Tsa2p might catalytically detoxify peroxynitrite. One efficient and selective method to detect peroxynitrite is through the oxidation of dihydrorhodamine 123 to rhodamine 123 (10). We expressed His-tagged Tsa2p protein in E. coli and purified it to >90% homogeneity as assessed on non-reducing and reducing PAGE gel (Fig. 5A). We incubated the purified His-Tsa2p with 5 mM DTT and then removed DTT by gel filtration. When this preparation of reduced His-Tsa2p was added to the reaction containing peroxynitrite and dihydrorhodamine 123, we observed a pronounced inhibition of rhodamine 123 formation (Fig. 5B, curve 3). Notably, neither bovine serum albumin treated with DTT in the same way as His-Tsa2p (Fig. 5B, curve 1) nor oxidized His-Tsa2p preincubated with 5 mM H2O2 (curve 2) had a significant effect on peroxynitrite-mediated oxidation of dihydrorhodamine 123. Moreover, we demonstrated that the oxidation of His-Tsa2p by peroxynitrite (Fig. 5C, lanes 1 and 2) can be reversed by the addition of β-mercaptoethanol (lane 3). Because the appearance of dimeric Tsa2p probably reflects the formation of an interchain disulfide bond, peroxynitrite may oxidize Tsa2p reversibly on cysteine residues. Thus, Tsa2p acts as a peroxynitrite reductase in vitro.

**Fig. 4. Sensitivity of tsa2Δ mutants to peroxynitrite and SNP.** A, sensitivity to peroxynitrite. Cells were grown to saturation, diluted to an A595 of 0.2, and then treated with 1 mM peroxynitrite. B, sensitivity to SNP. Cells were grown to saturation, diluted to an A595 of 0.2, and then treated with 2 mM SNP. C, sensitivity of TSA1-transformed mutants to SNP. The tsalΔ, tsa2Δ, and tsalΔ tsa2Δ strains (curves 2–4) were transformed with a TSA1 expression plasmid driven by the TSA1 promoter. The transformants were compared with the mock-transformed BY4741 strain (curve 1). D, sensitivity of TSA2-transformed mutants to SNP. The tsalΔ, tsa2Δ, and tsalΔ tsa2Δ strains (curves 2–4) were transformed with a TSA2 expression plasmid driven by the TSA2 promoter. The transformants were compared with the mock-transformed BY4741 strain (curve 1). E, expression of Tsa1p or Tsa2p rescues the sensitivity to SNP. The tsalΔ tsa2Δ strain was transformed individually with either pTSA1-2 (curve 5) or pTSA2-1 (curve 6). The expression of Tsa2p from pTSA1-2 was driven by a TSA1 promoter (~1000 to +52 nucleotides). In pTSA2-1, a TSA2 promoter (~1000 to +52 nucleotides) was used to control the expression of Tsa1p.
6-fold higher than that of Tsa2p (16). Above we showed that the expression of either Tsa1p or Tsa2p in yeasts sufficiently protected cells from ROS and RNS. To further characterize the antioxidant activities of Tsa1p and Tsa2p, we compared their H₂O₂-removing activities in vivo.

For this experiment, we constructed inducible expression plasmids for Tsa1p and Tsa2p. A GAL1 promoter was used to control the expression of Tsa1p and Tsa2p in plasmids pTSA1 and pTSA2, respectively. The tsa1Δ tsa2Δ double mutant was transformed individually with the empty vector, pTSA1, and pTSA2. The expression of Tsa1p and Tsa2p was induced by transferring the yeasts to a medium containing galactose. The cells were treated with H₂O₂, and the fluorescent dye 2′,7′-dichlorofluorescein diacetate was added to chase the removal of H₂O₂. The DCF fluorescence reflects the relative levels of intracellular ROS. From the representative fields of cells under the fluorescence microscope (Fig. 6A) and from the quantitation based on a fluorimeter (Fig. 6B), the expression of either Tsa1p or Tsa2p led to a substantial reduction of DCF fluorescence, which reflects the removal of intracellular H₂O₂. Tsa1p appeared to be a more potent peroxidase in this assay. For all, the H₂O₂-scavenging activities of the two peroxiredoxins were comparable. These data implicate that both Tsa1p and Tsa2p are active peroxiredoxins in vivo.

Comparison of the Basal Transcriptional Activities of TSA1 and TSA2—The above data suggested that the TSA1 promoter might be stronger than the TSA2 promoter (Fig. 5, C–E). To formally compare their basal activities, we performed lacZ reporter assays. Reporter plasmids (2µ-based) driven by the TSA1 and TSA2 promoters (TSA1-lacZ and TSA2-lacZ) were transformed into the BY4741 strain. The β-galactosidase activity was assayed and compared. In this assay, the TSA1 promoter is about 3 times stronger than the TSA2 promoter (Fig. 7). Results from the semiquantitative RT-PCR analysis of the TSA1 and TSA2 transcripts in untransformed BY4741 cells lent further support to the notion that the basal transcriptional level of TSA1 is significantly higher (Fig. 7, inset).

Differential Regulation of TSA1 and TSA2 by ROS and RNS—While the basal expression levels of TSA1 and TSA2 were different (Fig. 7), both genes have been shown to be induced by H₂O₂ and diamide (16). To investigate the transcriptional regulation of the chromosomal TSA1 and TSA2 loci, we inserted the lacZ reporter immediately downstream of the chromosomal TSA1 and TSA2 promoters. The expression of the Tsa1p/Tsa2p was rescued by simultaneously introducing an extra copy of the TSA1/TSA2 promoter immediately upstream of the coding region. In this setting, the integrated single copy lacZ reporter may better reflect the transcriptional activities of the chromosomal TSA1 and TSA2 genes.

We compared the relative β-galactosidase activities of the TSA1-lacZ and TSA2-lacZ strains in the presence of H₂O₂, t-BHP, diamide, peroxynitrite, and SNP (Fig. 8). Interestingly, the activities of the TSA1 promoter did not change substantially in response to ROS or RNS (Fig. 8A). In most cases, the increase in transcriptional level was less than 50%. The stimulation by t-BHP was less than 2-fold. By sharp contrast, the induction of TSA2 promoter by H₂O₂, t-BHP, diamide, and peroxynitrite was much more dramatic, ranging from 5- to 11-fold (Fig. 8B). Similar results were obtained from strains transformed with 2µ-based TSA1-lacZ and TSA2-lacZ plasmids (data not shown). These data provide the evidence for differential regulation of TSA1 and TSA2 genes in response to ROS and RNS.

Compensational Activation of TSA2 in tsa1Δ Strain—To better understand the functional overlap between Tsa1p and Tsa2p, we asked whether TSA1 is activated in the tsa2Δ strain, and vice versa. We observed that the activity of TSA1-lacZ was only slightly increased in the tsa2Δ strain either in the absence or in the presence of H₂O₂ (Fig. 8A). The TSA1-lacZ activity was substantially reduced in the yap1Δ tsa2Δ strain, implying that the basal activation of TSA1 is mediated through the redox-regulated transcription factor Yap1p. In contrast, the TSA2-lacZ activity significantly increased in the tsa1Δ strain (Fig. 8B). We also noted that the TSA2-lacZ activity was lost almost completely in the yap1Δ tsa1Δ strain. One interpretation is that Yap1p is responsible for both the basal and the induced activation of TSA2.

To verify that the lacZ reporter activity reflects the authentic TSA1 and TSA2 genes, we performed semiquantitative RT-PCR to compare the relative amounts of TSA1 and TSA2 mRNA in BY4741, tsa1Δ, and tsa2Δ strains (Fig. 9C). Consistent with the results from the reporter assay, the TSA2 mRNA was more abundantly expressed in the tsa1Δ strain (compare lane 2 with lane 1 and lane 5 with lane 4). This compensational activation suggests that TSA2 may play a more important role when TSA1 is compromised.

DISCUSSION

In this study, we used a genetic approach (Fig. 1) to characterize the function and regulation of yeast peroxiredoxins.
TsA1p and TsA2p. The tsa2Δ and tsΔΔ tsa2Δ yeast strains were viable and indistinguishable from the parental wild type under aerobic conditions (Fig. 2). However, the disruption of TsA1 and/or TsA2 conferred hypersensitivity to RNS (Fig. 4) in addition to ROS (Fig. 3). In line with this, TsA2p acts as a peroxynitrite reductase to protect against peroxynitrite-mediated oxidation in vivo (Fig. 5). The in vivo H$_2$O$_2$-scavenging activities of TsA1p and TsA2p were comparable (Fig. 6), but the basal expression level of TSA1 was significantly higher (Fig. 7).

While the transcription of TSA2 was potently activated in response to ROS/RNS (Fig. 8B) and as a result of TSA1 disruption (Fig. 9), the expression of TSA1 was less responsive to stimuli (Fig. 8A). Our findings support the model in which TsA2p cooperated with the primary cytoplasmic peroxiredoxin TsA1p in the cellular defense against oxidative and nitrosative stress.

TsA2p Is a Functional Antioxidant Enzyme in Yeast—TsA2p is closely related to the well described TsA1p. The separation of these two peroxiredoxins probably arose from a gene duplication event after the speciation of budding yeast. A previous study (16) has described two unique characteristics of a TsA2-null mutant: the insensitivity to oxidants and the induction of growth retardation presented as G1 arrest. In addition, TsA2p has a low thioredoxin peroxidase activity in vitro (16). In the present work, we did not observe the slow growth phenotype in tsa2Δ strains (Fig. 2). We wondered how the particular genetic background of the tsa2Δ strain used in Ref. 16 or changes other than the loss of TsA2 might explain the different observations. We also presented several lines of evidence to support the notion that TsA2p is a functional antioxidant enzyme in vivo. First, the tsa2Δ and tsΔΔ tsa2Δ strains are more sensitive to ROS and RNS than the parental BY4741 and tsa1Δ strains, respectively (Figs. 3 and 4). Second, the expression of TsA2 driven by different promoters can partially or fully rescue the hypersensitivity to SNP caused by disruption of TsA1 and/or TsA2 (Fig. 4, D and E). Third, TsA2p acts as a peroxynitrite reductase in vitro (Fig. 5). Fourth, the overexpression of TsA2 under the control of the inducible GAL1 promoter can effectively scavenge H$_2$O$_2$ in the tsa1Δ tsa2Δ cells (Fig. 6). TsA2p appears to be a more active peroxidase in vivo (Fig. 6) than in vitro (16). It remains unanswered whether electron donors other than thioredoxin can support the peroxidase and peroxynitrite reductase activities of TsA2p and other peroxiredoxins. In this regard, cyclophilin A has recently been shown as a binding partner as well as peroxidase activator of mammalian peroxiredoxins (38). It would be of interest to see whether yeast cyclophilins might serve similar functions to support the antioxidant activities of peroxiredoxins. Last but not least, the...
expression of TSA2 was stimulated potently by ROS and RNS (Fig. 8). Our findings argue for an important role of Tsa2p in the antioxidant defense.

**Tsa1p and Tsa2p Protect Cells against RNS**—The disruption of TSA1 and/or TSA2 conferred susceptibility to peroxynitrite and SNP (Fig. 4, A and B). In addition, the expression of either TSA1 or TSA2 sufficiently reversed this phenotype (Fig. 5, C–E). Thus, we provide the first evidence that eukaryotic peroxiredoxins conferred resistance to RNS. This appears to be a biological function conserved in both prokaryotic and eukaryotic peroxiredoxins (11). In support of this, we demonstrate for the first time the peroxynitrite reductase activity of Tsa2p in vitro (Fig. 5). Thus, bacterial peroxiredoxin AphC (10), yeast Tsa2p (this study), and bovine peroxiredoxin VI (also known as 1-Cys peroxiredoxin; Ref. 39) can act as peroxynitrite reductase to directly protect cells against peroxynitrite-mediated oxidations. Since these three peroxiredoxins are from different species and different subfamilies, it is tempting to assume that most if not all peroxiredoxins might conserve the same property in the cellular defense against RNS.

**Differential Expression and Cooperation of Tsa1p and Tsa2p**—TSA1 and TSA2 are differentially regulated in yeasts. On one hand, the basal transcription level of TSA1 is significantly higher (Fig. 7). In support of this, we demonstrate for the first time the peroxynitrite reductase activity of Tsa2p in vitro (Fig. 5). Thus, bacterial peroxiredoxin AphC (10), yeast Tsa2p (this study), and bovine peroxiredoxin VI (also known as 1-Cys peroxiredoxin; Ref. 39) can act as peroxynitrite reductase to directly protect cells against peroxynitrite-mediated oxidations. Since these three peroxiredoxins are from different species and different subfamilies, it is tempting to assume that most if not all peroxiredoxins might conserve the same property in the cellular defense against RNS.

**Fig. 8.** Induction of TSA1 and TSA2 expression in response to oxidative or nitrosative stress. A, induction of the TSA1-lacZ mutant yeast. B, induction of the TSA2-lacZ mutant yeast. A single copy of the lacZ gene integrated into the yeast genome was driven by the chromosomal TSA1 or TSA2 promoter. Cells were cultured in YPD medium to log phase and treated with 0.5 mM H$_2$O$_2$, 1 mM t-BHP, 1 mM diamide, 0.5 mM peroxynitrite, or 2 mM SNP. β-galactosidase activity of the untreated control cells (column 1) was taken as 100%. Results are representative of three independent experiments, and error bars indicate S.E.
On the other hand, the transcription of TSA2 is induced substantially in response to ROS, RNS, and the absence of TSA1 (Figs. 8 and 9). This induction indicates that Tsa2p plays a particularly important role in the adaptation to oxidative and nitrosative stress. Our findings are generally consistent with results from several genome-wide proteomic or microarray analyses (40–42). The differential expression of Tsa1p and Tsa2p suggests that these two peroxiredoxins may fulfill their functions in different phases of the cellular response to stress.

The cooperation between Tsa1p and Tsa2p is supported by three lines of data. First, the tsa1Δ tsa2Δ strain is more sensitive to ROS (Fig. 3) and RNS (Fig. 4) than the tsa1Δ strain. These results support the additive action of Tsa1p and Tsa2p. Second, the expression of TSA2 is activated in response to the loss of TSA1 (Fig. 9). Third, the induced overexpression of TSA2 alone can sufficiently complement the loss of TSA1 (Fig. 4). Taken together, the differentially expressed yeast peroxiredoxins Tsa1p and Tsa2p serve similar functions, and they cooperate with each other in the cellular defense against oxidative and nitrosative stress.

Acknowledgments—We thank M. M. K. Tsui and D. K. Benfield for technical help with yeast genetics; T. K. Chin for technical advice in protein purification; Y. P. Ching and O. S. W. Wong for helpful discussions; L. Derr and E. A. Elon for reagents; and Y. P. Ching, A. C. S. Chun, O. S. W. Wong, and S. F. Chan for critical reading of the manuscript.

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Fig. 9. Compensational activation of Tsa2p in tsa1Δ cells depends on Yap1p. A, activity of TSA1-lacZ reporter plasmid in tsa2Δ cells. B, activity of TSA2-lacZ reporter plasmid in tsa1Δ cells. Results represent three independent experiments, and error bars indicate S.E. C, RT-PCR. RT-PCR was performed to verify the transcriptional levels of TSA1 and TSA2 genes. The housekeeping gene ORC5 was used as a control.
Yeast Peroxiredoxins Tsa1p and Tsa2p in Antioxidant Defense

Cooperation of Yeast Peroxiredoxins Tsa1p and Tsa2p in the Cellular Defense against Oxidative and Nitrosative Stress
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doi: 10.1074/jbc.M106846200 originally published online December 10, 2001

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