The Peroxiredoxin Tpx1 Is Essential as a H₂O₂ Scavenger during Aerobic Growth in Fission Yeast

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Submitted November 27, 2006; Revised March 16, 2007; Accepted March 26, 2007

Monitoring Editor: Thomas Fox

Peroxiredoxins are known to interact with hydrogen peroxide (H₂O₂) and to participate in oxidant scavenging, redox signal transduction, and heat-shock responses. The two-cysteine peroxiredoxin Tpx1 of *Schizosaccharomyces pombe* has been characterized as the H₂O₂ sensor that transduces the redox signal to the transcription factor Pap1. Here, we show that Tpx1 is essential for aerobic, but not anaerobic, growth. We demonstrate that Tpx1 has an exquisite sensitivity for its substrate, which explains its participation in maintaining low steady-state levels of H₂O₂. We also show in vitro and in vivo that inactivation of Tpx1 by oxidation of its catalytic cysteine to a sulfinic acid is always preceded by a sulfinic acid form in a covalently linked dimer, which may be important for understanding the kinetics of Tpx1 inactivation. Furthermore, we provide evidence that a strain expressing Tpx1.C169S, lacking the resolving cysteine, can sustain aerobic growth, and we show that small reductants can modulate the activity of the mutant protein in vitro, probably by supplying a thiol group to substitute for cysteine 169.

INTRODUCTION

Peroxiredoxins (Prxs) are a family of antioxidant enzymes that reduce hydrogen peroxide (H₂O₂) and/or alkyl hydroperoxides to yield water and/or alcohol, using reducing equivalents provided principally by thioredoxin. These H₂O₂ scavengers have been isolated from all kingdoms (Chae *et al.*, 1994b), with six mammalian isoforms distributed in different organelles (Rhee *et al.*, 2005b). Three types of structurally different Prxs have been described: 1-cysteine (Cys), 2-Cys, and atypical 2-Cys (for review, see Wood *et al.*, 2003b). All three types of Prxs are dimers in solution, and they all share the same catalytic mechanism, in which the peroxidatic Cys, located close to the N-terminal domain, is oxidized to a sulfinic acid by either H₂O₂ or alkyl hydroperoxides. In both types of 2-Cys Prxs, the sulfinic acid then reacts with the C-terminal (or resolving) Cys of the other subunit to form an intermolecular disulfide (classical 2-Cys Prxs), or with the C-terminal Cys of the same monomer to form an intramolecular disulfide (atypical 2-Cys Prxs). In both cases, the disulfides are specifically reduced by the thioredoxin and thioredoxin reductase system, with the exception of some prokaryotic Prxs, such as the *Escherichia coli* AhpC, which uses another protein, AhpF, for the regeneration of the reduced Prx. In the 1-Cys Prxs, the sulfinic acid is directly reduced to thiol, because there is no nearby Cys

available to form a disulfide bond; the source of the reducing equivalents for regenerating this thiol is not known, although glutathione (GSH) has been proposed to serve as the electron donor in this reaction (Kang *et al.*, 1998b).

Prx activity can be regulated by phosphorylation (Chang *et al.*, 2002), and possibly by changes in oligomerization states (Wood *et al.*, 2002, 2003b). Furthermore, some Prxs suffer an oxidation of one of their Cys residues, the peroxidatic Cys, to sulfinic acid upon exposure to high H₂O₂ doses, which temporarily inactivate these enzymes (Yang *et al.*, 2002; Woo *et al.*, 2003b; Wood *et al.*, 2003a). This hyperoxidation of 2-Cys Prx enzymes is reversible in cells (Woo *et al.*, 2003a,b). The enzymes named sulfiredoxin, found in *Saccharomyces cerevisiae* (Biteau *et al.*, 2003), *Schizosaccharomyces pombe* (Bozonet *et al.*, 2005; Vivancos *et al.*, 2005), and mammalian cells (Chang *et al.*, 2004), were isolated as the ATP-dependent reductases for Cys-sulfinic acid in Prxs. Another family of enzymes named sestrins has been shown to have a similar role in mammalian cells (Budanov *et al.*, 2004).

The main role of Prxs has classically been considered to be related to their peroxidase activity. Their affinity for H₂O₂, with published K_m values ~20 μM, is higher than that of other peroxide scavengers such as catalase or GSH peroxidase (Chae *et al.*, 1999; Rhee *et al.*, 2001), which suggests that Prxs could be efficient at removing low concentrations of H₂O₂. However, their temporary redox inactivation seems to suggest that Prx enzymes might have a role in signal transduction, with their inactivation shunt causing a temporary or local increase in H₂O₂ concentration that could then trigger antioxidant cascades (Rhee *et al.*, 2005a).

The first evidence for the participation of a Prx in signal transduction pathways came from studies of fission yeast antistress responses. In *S. pombe*, the Pap1 and Sty1 pathways constitute the key protective responses to oxidative stress. It has been recently reported that the only 2-Cys Prx in fission yeast, Tpx1, is the H₂O₂ sensor of the Pap1 pathway, its presence being essential for activation of the tran-

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E06-11-1039>) on April 4, 2007.

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Abbreviations used: DNPH, dinitrophenylhydrazine; DTT, dithiothreitol; GSH, glutathione; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; Prx, peroxiredoxin; TCA, trichloroacetic acid.

scription factor and its specific gene response. The Pap1 pathway is more sensitive to H_2O_2 than the mitogen-activated protein kinase Sty1 pathway: maximal activation of Sty1 requires a H_2O_2 concentration at least fivefold higher than that described to fully activate Pap1 (Quinn *et al.*, 2002; Vivancos *et al.*, 2004). As described above, 2-Cys Prxs such as Tpx1 undergo substrate-mediated inactivation, so high concentrations of H_2O_2 will temporarily inactivate Tpx1, postponing Pap1 activation; meanwhile, the Sty1 pathway will be fully functional under these conditions. Activated Sty1 is then required for reactivation of Tpx1, because synthesis of the Tpx1-reductase Srx1 during severe H_2O_2 stress is dependent on Sty1 (Bozonet *et al.*, 2005; Vivancos *et al.*, 2005).

Thus, Prxs have aroused a great deal of interest during recent years, and their emerging roles other than H_2O_2 scavenging—as redox signal transducers and molecular chaperones (Jang *et al.*, 2004; Chuang *et al.*, 2006)—is igniting a very interesting field of study. Indeed, Tpx1 was initially identified on the basis of its role in signal transduction to Pap1 in fission yeast. However, evidence from deletion mutants points to additional actions by this Prx. A strain lacking the *tpx1* gene is not viable (Vivancos *et al.*, 2005), whereas deletion of either *pap1* or *sty1* genes does not compromise cell viability, and cells lacking any of the oxidative stress signaling components only display phenotypes in the presence of extracellular stress (for review, see Ikner and Shiozaki, 2005). In this work, we demonstrate that Tpx1 is an essential H_2O_2 scavenger in *S. pombe*, with an extremely high affinity for its substrate and that this peroxidase activity is required only for aerobic growth. A mutant Tpx1 protein lacking the resolving Cys169 (Tpx1.C169S) is also able to support aerobic growth of cells expressing only this isoform, and small thiols such as dithiothreitol and GSH can provide the reducing equivalents to recycle oxidized Tpx1.C169S in vitro. This is the first eukaryotic 2-Cys Prx reported to scavenge H_2O_2 in vivo in the absence of its resolving Cys, in a manner similar to 1-Cys Prx enzymes.

MATERIALS AND METHODS

Yeast Strains

We used the wild-type strains 972 (*h⁻*), PN513 (*h⁻ leu1 ura4*), HM123 (*h⁻ leu1*), AV29 (*h⁺/h⁻ leu1/leu1 ura4/ura4 ade6-M210/ade6-M276*); our laboratory stocks) as well as other published strains such as TP108 (*h⁺ leu1 his2 ura4 pap1::ura4*; our laboratory stocks), NT224 (*h⁻ leu1 ura4 sty1-1*) (Miller *et al.*, 1995), and EA38 (*h⁻ leu1 srx1::kanMX6*) (Vivancos *et al.*, 2005). To construct *S. pombe* strains with specific loci deleted, we transformed some of the above-mentioned strains with linear fragments containing ORF::kanMX6, obtained by polymerase chain reaction (PCR) amplification with open reading frame (ORF)-specific primers and plasmid pFA6a-kanMX6 as a template. Following that strategy, we obtained strains AV25 (*h⁻ pap1::kanMX6*), AV18 (*h⁻ sty1::kanMX6*) and AM42 (*h⁻ leu1 his2 ura4 pap1::ura4 sty1::kanMX6*). To isolate cells deleted in *sod1*, we transformed the wild-type strain PN513 with a PCR-amplified *sod1::lacZ::kanMX6* fragment, obtained by PCR amplification with ORF-specific primers and plasmid AY017 as a template (plasmid AY017 is a pREP3x [Maundrell, 1993] derivative containing a *lacZ::kanMX6* insert). Transformed cells were grown under anaerobic conditions and selected by their ability to grow in Kanamycin-containing plates. The strain generated was named MS1 (*h⁻ leu1 ura4 sod1::lacZ::kanMX6*). To isolate haploid cells deleted in *tpx1*, we transformed the diploid strain AV29 with a PCR-amplified *tpx1::kanMX6* fragment, yielding strain AV36dip (*h⁺/h⁻ leu1/leu1 ura4/ura4 ade6-M210/ade6-M276 tpx1::kanMX6/tpx1*). Haploid cells were isolated by standard genetic techniques (Moreno *et al.*, 1991), spread in Kanamycin-containing plates and grown anaerobically (see below). This yielded strain AV42 (*h⁺ leu1 ura4 ade6-M210 tpx1::kanMX6*). Plasmids p145 (*ptpx1::tpx1*), p145.C48S (*ptpx1::tpx1.C48S*), and p145.C169S (*ptpx1::tpx1.C169S*), containing the promoter of *tpx1* fused to the wild-type or mutant ORF of *tpx1* (Vivancos *et al.*, 2005), were linearized and introduced at the *leu1* locus of strain AV42, yielding strains AV49, AV49.C48S, and AV49.C169S. To construct the strain AV49.C169S disrupted in *cys1a*, we crossed it with strain *cys1aΔ* (*h⁻ leu1-32 ura4-C190T cys1a::ura4*) (Fujita and Takegawa, 2004). An equal volume of each strain was mixed under starvation conditions for nitrogen source. After 2 d at 25°C, the mating mixture was suspended in 0.7 ml of sterile distilled water

with 0.3% β -glucuronidase (Sigma-Aldrich, St. Louis, MO) and incubated at 25°C overnight. The resulting spores were washed and resuspended in water, spotted on YE5S plates supplemented with Cys and G-418 (Geneticin; Invitrogen, Paisley, United Kingdom) and streaked for single colonies. Kanamycin-resistant cells with auxotrophy for Cys were isolated and confirmed by PCR and immunodetection of trichloroacetic acid (TCA)-based protein extracts. The resulting strain was named MJ1.

Anaerobic Growth Conditions and H_2O_2 Sensitivity Assay

Anaerobic liquid cultures were grown in flasks filled to the top with medium. Cells were grown at 30°C without shaking. To grow cells in solid media in an anaerobic environment, we incubated the plates at 30°C in a tightly sealed plastic bag containing a water-activated Anaerocult A (Merck, Darmstadt, Germany) sachet. To analyze sensitivity to H_2O_2 on plates, *S. pombe* strains were grown anaerobically in liquid rich media to an OD₆₀₀ of 0.5. Cells were then diluted in water, and the indicated number of cells in 4 μ l was spotted onto rich media. The spots were allowed to dry, and the plates were incubated at 30°C in aerobic and anaerobic conditions, in the presence or absence of H_2O_2 , for 3–4 d.

Plasmids

To express *S. pombe* proteins in *Escherichia coli*, the ORFs coding for Tpx1, Tpx1.C48S, and Tpx1.C169S were digested from plasmids p123.41x, p123.41x.C48S, and p123.41x.C169S with BamHI and SmaI, and they were subcloned into a modified glutathione S-transferase (GST)-tagging fusion vector pGEX-2T-TEV that encoded a TEV protease cleavage site between the tag and the cloned ORF, digested with the same restriction enzymes. The resulting plasmids were called p210, p210.C48S, and p210.C169S. The *trr1* ORF was amplified from an *S. pombe* cDNA library by using specific primers containing BamHI and EcoRI restriction sites, and then it was cloned into the pGEX-2T-TEV expression vector digested with the same restriction enzymes, yielding plasmid p205. The *trx1* ORF was amplified from genomic DNA using specific primers for the gene and containing BamHI and SmaI restriction sites. The fragment was cloned into pGEX-2T-TEV cleaved with BamHI and SmaI, yielding plasmid p206. Once PCR amplified, all ORFs in these expression vectors were sequenced.

Preparation of *S. pombe* Extracts to Measure Protein Carbonylation

Protein carbonylation was detected after in vitro derivatization of oxidized proteins with 2,4-dinitrophenylhydrazine (DNPH) (Levine *et al.*, 1994; Cabiscoll and Levine, 1995). Cells (50 ml) were grown aerobically over 12 h in rich media, harvested at an OD₆₀₀ of 0.5, and resuspended in carbonylation buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.05% NP-40, 2 mM phenylmethylsulfonyl fluoride [PMSF], 5 mM benzamidine, 0.16 mg/ml aprotinin) and lysed by vortexing after the addition of glass beads. The protein extracts were then centrifuged to eliminate cell debris. Protein concentration was determined using the Bradford protein assay (Bio-Rad, Hercules, CA). Protein concentration was adjusted to a 4–10 mg/ml range with carbonylation buffer, and a concentrated 12% solution of SDS was added to 10 μ g of total protein, to reach a final SDS concentration of 6%, and incubated for 3 min at 100°C. One volume of 10 mM DNPH in 10% trifluoroacetic acid was added to 1 volume of the sample at 25°C. The reaction was run for 10 min and stopped by the addition of 1 volume of 2 M Trizma base, 10% glycerol, and 15% 2-mercaptoethanol. Samples were separated electrophoretically in 10% SDS-polyacrylamide gel electrophoresis (PAGE) gels and carbonylated proteins were immunodetected using a polyclonal anti-2,4-dinitrophenyl (DNP) antibody (Sigma-Aldrich). As a loading control, Sty1 was detected using polyclonal anti-Sty1 antiserum raised against an *E. coli* fusion protein of GST-Sty1, following standard rabbit immunization procedures. The anti-DNP and anti-Sty1 immunoblots were scanned, and quantification of carbonylated proteins was performed using the ImageQuant 5.2 program (GE healthcare, Little Chalfont, Buckinghamshire, United Kingdom).

Purification of Recombinant Tpx1, Thioredoxin (Trx1), and Thioredoxin Reductase (Trr1) Proteins for In Vitro Assays

Bacteria strain FB810 (Benson *et al.*, 1994) transformed with the pGEX-2T-TEV derivatives were inoculated into Luria broth with 100 μ g/ml ampicillin and incubated at 37°C for ~16 h with vigorous shaking. The overnight cultures were diluted 25-fold into 400 ml of fresh medium and incubated at 37°C until the culture reached an optical density of 0.8 at 600 nm. Isopropyl- β -thio-*D*-galactoside (IPTG) was then added to a final concentration of 0.5 mM and shaking continued at 25°C for 4 h. The cells were then harvested, and pellets were resuspended in 10 ml of STET extraction buffer (50 mM Tris-HCl, pH 8.0, 150 mM sodium chloride, 1 mM EDTA, pH 8.0, 1% Triton X-100, and 2 mM PMSF) and broken by sonication. Debris and unbroken cells were removed by centrifugation. Supernatants containing our GST-tagged fusion proteins were then incubated with GSH-Sepharose 4B beads (GE Healthcare) for 1 h at 4°C. The beads were then washed three times with NET-N (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM sodium chloride, 0.5% NP-40, and 2 mM PMSF) and once with TEV cleavage buffer (10 mM Tris-HCl, pH 8.0, 150 mM sodium

chloride, 0.1% NP-40, and 0.5 mM EDTA). We could then release the GST-tagged protein with GSH-containing elution buffer (100 mM Tris HCl, pH 8.0, 120 mM NaCl, and 20 mM GSH). Alternatively, we also released the *S. pombe* proteins without the GST tag from the Sepharose beads by incubating a 200- μ l bed volume of beads with 10 μ g of TEV protease (Invitrogen, Carlsbad, CA) overnight at 4°C. Size differences among GST-containing or untagged protein samples Tpx1, Trx1, and Trx1 were observed by electrophoretic separation on 15% denaturing polyacrylamide gels and Coomassie staining.

Peroxidase Activity Assays

NADPH oxidation was monitored as a decrease in optical density at 340 nm by using a Ultrospec 3100 *pro* UV/Visible spectrophotometer (GE Healthcare) in a 500- μ l reaction mixture containing 50 mM HEPES-NaOH, pH 7.0, 0.25 mM NADPH, 6 μ g of Trx1, 20 μ g of Tpx1, 1 μ g of Tpx1, and H₂O₂. Reactions were started by the addition of the indicated concentrations of H₂O₂. As peroxidase activity of Tpx1 undergoes substrate-mediated inactivation, an initial linear portion of absorbance change (10 s) was used for the calculation of peroxidase activity, as described previously (Koo *et al.*, 2002).

To determine the H₂O₂ detoxification driven by Tpx1, Tpx1.C48S, and Tpx1.C169S in the presence of small thiols, we incubated 5–20 μ g of enzyme with the Trx system (consisting of the same reaction mixture as described in the previous paragraph), 10 or 100 mM dithiothreitol (DTT) (in 50 mM HEPES-NaOH, pH 7.0), or 50 μ M GSH (in 100 mM Tris-HCl, pH 8.8, to avoid acidification of the assay mixture). We started the reaction by the addition of 50 μ M H₂O₂, and 150- μ l aliquots were taken at different time points and stopped by the addition of 20 μ l of 100% TCA. Samples were then centrifuged to eliminate proteins, and 100 μ l of the supernatant were used to determine the remaining H₂O₂ concentration. This was achieved by addition of 27 μ l of 10 mM ferrous ammonium sulfate and 13.5 μ l of 2.5 M potassium thiocyanate. The red-colored ferrithiocyanate complex, which occurs as a result of the oxidation of Fe(II) by H₂O₂, was quantified by measuring the OD at 480 nm and was compared with H₂O₂ standards.

Preparation of *S. pombe* TCA Extracts and Immunoblot Analysis

For *in vivo* redox state analysis of Tpx1, *S. pombe* cultures (5 ml) at an OD₆₀₀ of 0.5 were pelleted just after the addition of 100% TCA, to a final concentration of 10%, and washed in 20% TCA. The pellets were lysed by vortexing, after the addition of glass beads and 12.5% TCA. Cell lysates were pelleted, washed in acetone, and dried. Alkylation of free thiols was performed by resuspension of the pellets in 50 μ l of a solution containing 75 mM iodoacetamide, 1% SDS, 100 mM Tris-HCl, pH 8.0, and 1 mM EDTA, and incubation at 25°C for 15 min. Alkylated samples were diluted fivefold and electrophoretically separated by reducing SDS-PAGE, as indicated, and proteins were immunodetected using polyclonal anti-Tpx1 antiserum raised against an *E. coli* fusion protein of GST-Tpx1, following standard rabbit immunization procedures. For the detection of sulfinylated Tpx1, the undiluted alkylated samples were separated by nonreducing SDS-PAGE and immunodetected with anti-peroxiredoxin-SO₃ antibody (LabFrontier, Seoul, South Korea).

RESULTS

Tpx1 Is Not Required for Growth under Anaerobic Conditions

Using classic genetic techniques, we could not isolate haploid *S. pombe* cells carrying a deletion of the *tpx1* gene, and we therefore generated a conditional knockout strain with Tpx1 expression under the control of a thiamine-repressible *nmt* promoter (Vivancos *et al.*, 2005). To test whether the essential role of Tpx1 in fission yeast was due to its peroxidase activity, we attempted to isolate haploid cells carrying the *tpx1* deletion from diploid cells, in which one of the *tpx1* loci is substituted by the kanamycin cassette, by growing them under anaerobic conditions inside sealed Anaerocult sachets. The chemical mixture inside the sachets contains components that chemically bind oxygen rapidly and effectively, creating an oxygen-free atmosphere. Positive clones were isolated, grown anaerobically in liquid, and doubly tested for lack of Tpx1 and their inability to oxidize Pap1 in response to H₂O₂ (data not shown). Deletion of *tpx1* increased the sensitivity of cells to aerobic growth, but cells grew as wild type in the absence of oxygen (Figure 1A). On the contrary, cells lacking Pap1, Sty1, or both grew as efficiently under aerobic or anaerobic conditions (Figure 1A).

Figure 1. Tpx1 has an essential role in *S. pombe* as a H₂O₂ scavenger. (A) Survival of wild-type, $\Delta tpx1$, $\Delta pap1$, $\Delta sty1$, and $\Delta pap1 \Delta sty1$ strains in response to aerobic growth. Strains HM123 (WT), AV42 ($\Delta tpx1$), TP108 ($\Delta pap1$), NT224 ($\Delta sty1$), and AM42 ($\Delta pap1 \Delta sty1$) were grown anaerobically in rich media to a final OD₆₀₀ of 0.5, and the number of cells indicated at the top of the panels was spotted in duplicate onto YE5S plates and incubated at 30°C for 3–4 d in aerobic or anaerobic conditions. (B) Protein carbonylation generated during aerobic growth. Strains 972 (WT), MS1 ($\Delta sod1$) and AV42 ($\Delta tpx1$) were grown aerobically for 12 h to a final OD₆₀₀ of 0.5, and protein carbonylation was detected by reaction of carbonyl groups with DNP, followed by SDS-PAGE and Western blot analysis by using anti-DNP antibodies (α -DNP, top) or anti-Sty1 antibodies as a loading control (α -Sty1, bottom). -Fold induction numbers, obtained from the same blots, are the ratio of the absolute scan numbers for the indicated band (*) and the corresponding amount of Sty1, and they relate to the values of the wild-type strain (see *Materials and Methods*).

Aerobic conditions were toxic to $\Delta tpx1$ cells. To test whether these cells were suffering from high levels of intrinsic oxidative stress when grown in the presence of oxygen, we measured the levels of protein carbonylation. Redox-cycling cations such as reduced iron or copper can bind to proteins, and, with the aid of further H₂O₂ attack, they can transform side-chain amino groups of several basic amino acids, such as lysine, arginine, proline, or histidine, into carbonyls. These carbonyl groups can be derived *in vitro* with DNP, a modification that can be immunodetected with anti-DNP antibodies (see *Materials and Methods*). Under aerobic growth conditions, protein carbonylation in $\Delta tpx1$ cells was significantly higher than in wild-type cells (Figure 1B). Indeed, extracts of cells lacking Tpx1 contained as many protein carbonyls as those of a strain lacking the *sod1* gene, which encodes the cytosolic superoxide dismutase; this strain is also only viable when grown under anaerobic conditions (data not shown).

Tpx1 Is a Peroxidase with Very High Affinity for H₂O₂

In most cell types, Prxs are not essential for aerobic growth. However, the lack of Tpx1 seems to drive cells grown in the presence of oxygen toward a basal oxidative stress, resulting in growth arrest. We decided to test whether Tpx1 scavenges the majority of endogenous H₂O₂ in *S. pombe* by assaying its capacity to decompose low concentrations of peroxides *in vitro*. The enzymatic activity of Prxs can be measured by coupling them to Trx1 and Trx1 in the presence of NADPH, following the reaction illustrated in Figure 2A. Traditionally, to assay the activity of eukaryotic Prxs, the three protein components of this reaction were purified from cell extracts by a series of multiple purification steps. We have developed an alternative strategy, in which Tpx1, Trx1, and Trx1 of *S. pombe* are purified from *E. coli* cells. We constructed expression vectors harboring full-length *tpx1*, *trx1*, and *trr1* genes

Figure 2. Determination of in vitro Tpx1 peroxidase activity in the presence of different concentrations of H_2O_2 . (A) Mechanism of peroxide reduction by Tpx1 coupled to NADPH oxidation. (B–D) NADPH oxidation coupled to the reduction of H_2O_2 by recombinant Trx1, Trx1, and Tpx1. The decrease of absorbance at 340 nm was monitored at room temperature in a 500- μl peroxidase reaction mixture containing 6 μg of Trx1, 20 μg of Trx1, and 1 μg of Tpx1 and low concentrations ($<50 \mu\text{M}$; B), medium concentrations (50–200 μM ; C), and higher doses of H_2O_2 (1–25 mM; D). The background of NADPH oxidation in the absence of peroxide is also present (control). (E–G) Effects of H_2O_2 concentration on the peroxidase activity of Tpx1 and Trx1. (E) Global Tpx1 peroxidase activity (micromoles of NADPH per minute per milligram) at different concentrations of H_2O_2 . Reactions were performed as described for B to D. An initial linear portion of absorbance change (10 s) was used for the calculation of peroxidase activity. (F) Effects of H_2O_2 concentration on the peroxidase activity of Trx1 and Trx1 in the absence of Tpx1. Experiments were performed as described in E, but in the absence of Tpx1. (G) Net Tpx1 peroxidase activity (micromoles of NADPH per minute per milligram) at different concentrations of H_2O_2 . The results from F were subtracted from those of E, yielding the Tpx1-dependent peroxidase activity of the reaction mixture.

fused to the GST-coding gene. The fusion proteins (GST-Tpx1, GST-Trx1, and GST-Trx1) were purified almost to homogeneity from *E. coli* extracts by affinity binding to GSH-beads. The GST tag interfered with the activity of some of the purified proteins, so we cleaved it with TEV protease, yielding highly purified recombinant Tpx1, Trx1, and Trx1. All the assays described hereafter were performed using these purified *E. coli*-expressed proteins, even though identical activities were detected in extracts of *S. pombe* cells overexpressing the wild-type or mutant Tpx1 (data not shown).

We measured Tpx1 activity by following NADPH oxidation at 340 nm in a reaction mixture containing NADPH, Trx1, Trx1, Tpx1, and varying concentrations of H_2O_2 . At low peroxide concentrations (5–50 μM ; Figure 2B), the rates of NADPH oxidation (the slope at the different time points) did not change with time and was proportional to H_2O_2 concentration, as expected for enzymes following Michaelis kinetics. As described for other eukaryotic Prxs, at intermediate H_2O_2 concentrations (50–200 μM), the rate of NADPH oxidation decreased with time, and the rate of this decrease was enhanced at higher H_2O_2 concentrations (Figure 2C). Surprisingly, high peroxidase activities were again achieved at peroxide concentrations above 1 mM, with the slopes at the different time points remaining constant at these H_2O_2

concentrations (Figure 2D). We then used the initial linear portion of absorbance change (10 s) to calculate the peroxidase activity at different concentrations of H_2O_2 , and the results are shown in Figure 2E. An exact K_m value could not be accurately established because the changes in A_{340} were not large enough to allow determination of initial rates. Furthermore, at saturating concentrations of H_2O_2 ($>50 \mu\text{M}$), the substrate-mediated inactivation of Tpx1 distorts the typical Michaelis kinetics. Nevertheless, applying the Lineweaver-Burk double-reciprocal plot to the Tpx1 activities at H_2O_2 concentrations from 5 to 50 μM , we could calculate that the apparent K_m of Tpx1 for H_2O_2 is ~ 2 –3 μM . The H_2O_2 -dependent inactivation of Tpx1 occurred at peroxide concentrations $>50 \mu\text{M}$. Thioredoxin has been suggested to act as a H_2O_2 peroxidase by itself (Hirota *et al.*, 2002); so, to measure the H_2O_2 -scavenging activity of Trx1 alone, we monitored NADPH oxidation by Trx1-Trx1 in the absence of Tpx1. As shown in Figure 2F, Trx1 was able to reduce H_2O_2 , but with a K_m value of at least 10 mM. This low affinity suggests that Trx1 is unlikely to contribute to H_2O_2 scavenging in vivo. Subtraction of the activity values for Trx1 (Figure 2F) from those of Tpx1 in the presence of the Trx1 system (Figure 2E) allowed us to calculate values for Tpx1 activity, which are shown in Figure 2G.

Figure 3. Formation of Cys-sulfinic acid occurs in a Tpx1 covalent dimer. (A) Cys-sulfinic acid dimer formation in vitro. The hyperoxidation of purified Tpx1 was monitored before (–) and after (+) incubation of the enzyme with Trx1/Trr1/NADPH and the indicated concentrations of H_2O_2 . The reaction conditions were as described for Figure 2. Samples were TCA precipitated, resuspended in loading buffer, and immunodetected with anti-Tpx1 antibodies (bottom) or anti-peroxiredoxin- SO_3 antibodies

(top) after nonreducing or reducing SDS-PAGE, respectively. (B) Formation of Tpx1- SO_2H dimer and monomer in response to different concentrations of H_2O_2 in vivo. The redox state (dimer vs. monomer) of sulfinic acid formation in Tpx1 was determined in TCA extracts from wild-type strain 972, treated or untreated for 5 min with the indicated concentrations of H_2O_2 . Tpx1- SO_2H monomer versus Tpx1- SO_2H dimer were immunodetected after nonreducing SDS-PAGE by using anti-peroxiredoxin- SO_3 antibodies, and they are indicated with arrows. A nonspecific, H_2O_2 -independent band was only present occasionally, and it is indicated with an asterisk (*). (C) Kinetics of Tpx1- SO_2H dimer and monomer formation in response to 1 mM H_2O_2 in vivo. Same as described in B, after treatment of wild-type strain 972 with 1 mM H_2O_2 for the times indicated in the figure.

Overoxidation of Cys-48 of Tpx1 to a Cys-Sulfinic Acid Occurs at a Covalent Tpx1 Dimer Interface

Once established that Tpx1 suffers H_2O_2 -mediated inactivation in vitro (Figure 2G), we decided to study whether such inactivation was concomitant with the oxidation of Cys-48 to the sulfinic acid state. To detect hyperoxidized Tpx1, we used commercial polyclonal antibodies against a sulfonated peptide encompassing the active site of mammalian Prx, which is identical in sequence to that of *S. pombe* Tpx1. These antibodies recognize sulfinic (and sulfonic) forms of Prxs and permit the detection of sulfinylated Prx enzymes in extracts from H_2O_2 -treated cells with high sensitivity and specificity (Woo *et al.*, 2003b). The presence of oxygen during the protein purification procedure was able to induce the formation of a disulfide bond in Tpx1 (data not shown). Nevertheless, as described for other Prxs, H_2O_2 is not sufficient to cause hyperoxidation of the peroxidatic Cys-SH of Tpx1 to Cys- SO_2H (Figure 3A). Instead, all catalytic components (Trx1, Trr1, and NADPH) are required. To our surprise, we detected two bands on immunoblots of in vitro hyperoxidized Tpx1 separated by nonreducing SDS-PAGE: a faster migrating band corresponding in size to a Tpx1- SO_2H monomer, and another band migrating as a disulfide-linked Tpx1- SO_2H dimer (Figure 3A). This dimeric form is DTT sensitive (data not shown), and it is not an artifact of purification procedures: immunoblot analysis of *S. pombe* extracts showed that the relative proportion of the monomeric and dimeric sulfinic acid forms depends on the H_2O_2 concentration (Figure 3B); in these blots, a nonspecific, H_2O_2 -independent band was present only occasionally, and it is indicated with an asterisk. As shown in Figure 3B, a small amount of Tpx1- SO_2H dimer is formed 5 min after exposure of cells to 0.2 mM H_2O_2 , a concentration known to yield catalytically active Tpx1 dimers (Bozonet *et al.*, 2005; Vivancos *et al.*, 2005). On exposure to higher H_2O_2 concentrations, Tpx1 accumulated in the form of a hyperoxidized monomer (Figure 3B). To ascertain whether the formation of a Tpx1- SO_2H dimer always precedes the accumulation of an inactive Tpx1- SO_2H monomer, we performed an in vivo time course at 1 mM H_2O_2 , which is the minimum concentration of peroxide known to fully inactivate Tpx1 (Vivancos *et al.*, 2005). As seen in Figure 3C, a Tpx1- SO_2H dimer can be detected only 5 s after exposure of the cell culture to 1 mM H_2O_2 , which is then gradually transformed into the monomeric form.

Cys-169 of Tpx1 Is Not Required for Its Peroxidatic Activity In Vivo

Tpx1 contains two Cys residues, Cys-48 and Cys-169, both of which are essential for its role in redox signal transduction

to Pap1 (Bozonet *et al.*, 2005; Vivancos *et al.*, 2005). Tpx1 belongs to the typical 2-Cys Prx family. Members of this family are H_2O_2 scavengers, and the peroxidase reaction of these enzymes also requires both conserved Cys residues, because the oxidized enzyme intermediate generated during the catalytic cycle is a dimer in which the subunits are linked by one or two intermolecular disulfide bonds between the peroxidatic and the resolving Cys of the other subunit (for review, see Wood *et al.*, 2003b). We analyzed what effect mutations of each one of the two Cys residues would have on cell viability. Surprisingly, a protein lacking the resolving Cys, Tpx1.C169S, was able to support aerobic growth of *S. pombe* cells (Figure 4A, middle), whereas the absence of the peroxidatic residue yielded a protein unable to sustain growth in the presence of oxygen. This result indicates that Tpx1.C169S is able to efficiently scavenge the H_2O_2 generated during aerobic growth. We subsequently measured protein carbonylation as an index of intrinsic oxidative stress. Under aerobic growth conditions the levels of protein carbonylation in extracts from cells expressing Tpx1.C169S were comparable with those of wild-type cells, whereas extracts from cells expressing Tpx1.C48S or lacking Prx enzymes showed significantly higher protein carbonylation (Figure 4B). Nevertheless, the ability of Tpx1.C169S to decompose high concentrations of peroxides is limited, because expression of Tpx1.C169S decreased cell tolerance to extracellular H_2O_2 to a greater extent than that of $\Delta pap1$ or $\Delta srx1$ cells (Figure 4A, right).

As stated above, inactivation by sulfinic acid formation can only occur in Prxs when the protein is engaged in the catalytic cycle. We were able to detect Tpx1.C169S- SO_2H in extracts from cells treated with H_2O_2 (Figure 5A), which demonstrates that Tpx1.C169S is catalytically active in vivo. As determined experimentally, disulfides in typical 2-Cys Prxs are specifically reduced by thioredoxin, but they can also be reduced in vitro by a small molecular thiol, such as DTT (Chae *et al.*, 1994a). Indeed, it has been demonstrated that DTT can support full activity in vitro of a Prx lacking its resolving Cys, whereas the Trx system cannot (Chae *et al.*, 1994a). According to the model proposed by Rhee and colleagues, a molecule of DTT could replace the resolving Cys in the formation of a disulfide with the peroxidatic Cys-SOH, and subsequently another molecule of DTT could reduce the mixed disulfide. We assayed the activity of wild-type and mutated Tpx1 by measuring the removal of H_2O_2 from the reaction mixture by using the ferrithiocyanate method (see *Materials and Methods*). Wild-type Tpx1 was able to scavenge peroxides in the presence either of the Trx1 system or, weakly, of DTT (Figure 5B). The Tpx1-C48S mutant enzyme was completely inactive under all condi-

Figure 4. The resolving Cys of Tpx1 is not essential for aerobic growth. (A) Survival of different strains in aerobiosis. Strains 972 (WT), AV25 ($\Delta pap1$), AV18 ($\Delta sty1$), EA38 ($\Delta srx1$), AV49 ($\Delta tpx1 tpx1$), AV49.C48S ($\Delta tpx1 tpx1.C48S$), and AV49.C169S ($\Delta tpx1 tpx1.C169S$) were grown anaerobically in rich media (YE5S) to a final OD₆₀₀ of 0.5, and the number of cells indicated at the top of the panels was spotted onto YE5S plates containing or not containing 0.8 mM H₂O₂. Plates were incubated at 30°C for 3–4 d in aerobic or anaerobic conditions, as indicated. (B) Protein carbonylation generated during aerobic growth. Strains 972 (WT), AV42 ($\Delta tpx1$), AV49.C48S ($tpx1.C48S$), and AV49.C169S ($tpx1.C169S$) were grown aerobically for 12 h to a final OD₆₀₀ of 0.5, and protein carbonylation was detected by reaction of carbonyl groups with DNP, followed by SDS-PAGE and Western blot analysis by using anti-DNP antibodies (α -DNP; top) or anti-Sty1 antibodies as a loading control (α -Sty1; bottom). Quantification data (-fold induction) was obtained as described in Figure 1B.

tions assayed. The C169S mutant is able to scavenge H₂O₂ in the presence of either the Trx1 system or DTT. Moderate concentrations of GSH were also able to support Tpx1 and Tpx1.C169S activity. In an attempt to modulate the intracellular concentration of small reductants in vivo and regulate the activity of the Tpx1.C169S protein in aerobically growing cultures, we isolated *S. pombe* cells expressing the mutant protein in a $\Delta cys1a$ background. The *cys1a* gene product, Cys synthase, is essential for the synthesis of L-Cys, and there-

fore $\Delta cys1a$ cells are auxotrophic for the amino acid (Fujita and Takegawa, 2004). Therefore, decreasing the extracellular supply of L-Cys diminishes the intracellular availability of the amino acid in $\Delta cys1a$ cells, thereby limiting the pool of intracellular GSH. We compared the sensitivity of cells lacking or possessing the *cys1a* gene and expressing wild-type or mutant Tpx1 under Cys-limiting conditions (0.08 mg/ml; Figure 5C). As a loading control, we incubated one plate under anaerobic conditions (Figure 5C, left). The double mutant $\Delta cys1a tpx1.C169S$ displayed decreased viability in comparison with the single mutants $\Delta cys1a$ or $tpx1.C169S$. Both the $\Delta cys1a$ and the double mutant had a lower intracellular GSH concentration than cells possessing the *cys1a* gene (5-fold decrease; data not shown). The lower GSH concentration in the $\Delta cys1a tpx1.C169S$ strains is probably not sufficient to support the enzymatic activity of the mutant Tpx1 protein, and H₂O₂ steady-state concentration rises under those conditions, generating oxidative stress and leading to cell death.

Figure 5. Tpx1.C169S has peroxidase activity in vitro and in vivo. (A) Tpx1.C169S can be hyperoxidized to the sulfinic acid form in vivo. The redox state of Tpx1 (with TCA extracts resolved in reducing SDS-PAGE) was determined from strains 972 (expressing wild-type Tpx1) and AV49.C169S (expressing Tpx1.C169S) treated or untreated with 1 mM H₂O₂ for 5 min. Western blot analysis permitted the detection of Tpx1-SO₂H (using commercial anti-peroxiredoxin-SO₃ antibodies) or total Tpx1 (using anti-Tpx1 antibodies). (B) In vitro H₂O₂ detoxification by Tpx1.C169S in the presence of small thiols. The micromoles of consumed H₂O₂ per minute and per milligram of Prx in a reaction mixture containing wild-type or mutant Tpx1 and the Trx1 system, GSH, or DTT, as indicated, were determined with the ferrithiocyanate method as described in *Materials and Methods*. (C) Tpx1.C169S in vivo function is affected by deletion of the *cys1a* gene. Strains 972 (WT), AV49.C169S ($tpx1.C169S$), AV42 ($\Delta tpx1$), $\Delta cys1a$, and MJ1 ($\Delta cys1a tpx1.C169S$) were grown anaerobically in rich media (YE5S) to a final OD₆₀₀ of 0.5, and the number of cells indicated at the top of the panels was spotted onto MM plates containing 0.08 mg/ml Cys, under aerobic (right) or anaerobic (as a loading control; left) conditions. Plates were incubated at 30°C for 4–6 d.

DISCUSSION

Several roles have been ascribed to peroxiredoxins in vivo: H₂O₂ scavengers (Rhee *et al.*, 1994), components of signal transduction pathways (Veal *et al.*, 2004; Bozonet *et al.*, 2005; Vivancos *et al.*, 2005) and chaperones after heat stress (Jang *et al.*, 2004; Chuang *et al.*, 2006). The only 2-Cys Prx of *S. pombe*, Tpx1, has been previously reported to participate in signal transduction in response to mild extracellular oxidative stress, but we have demonstrated in the present study that it also plays an essential role as a scavenger of H₂O₂ generated during oxidative metabolism. $\Delta tpx1$ cells shifted from anaerobic to aerobic conditions suffered growth arrest; this was probably a consequence of intracellular oxidative stress, as indicated by their elevated levels of protein carbonylation. Steady-state levels of H₂O₂ in aerobically growing cells are reported to be $\sim 10^{-7}$ M (Seaver and Imlay, 2001). As shown for other Prxs, the V_{max} of Tpx1 indicates that this is a slow detoxifying enzyme compared with catalases or GSH peroxidases; but its high-affinity for H₂O₂ (with a $K_m \sim 2 \mu\text{M}$) and its abundance (0.1–1% of total soluble proteins in *S. pombe*; data not shown) indicate that Tpx1 is very efficient at removing low concentrations of H₂O₂.

Other model organisms also have been shown to depend on Prxs for survival under aerobic conditions, such as the oxygen-sensitive microaerophile *Helicobacter pylori* (Baker *et al.*, 2001). In the yeast *S. cerevisiae*, although cells lacking all five Prxs are still able to grow aerobically, they are hypersensitive to oxidative stress and are genomically unstable

(Wong *et al.*, 2004). The fission yeast *S. pombe* has a limited set of detoxifying enzymes compared with budding yeast. The reason for such a limited number of antioxidants is unknown, but this may explain why deletion of only one gene has such dramatic consequences for the fission yeast but not for *S. cerevisiae*.

Previous studies have demonstrated that both Cys residues of Tpx1 are required for Pap1 activation in response to low H₂O₂ stress (Bozonet *et al.*, 2005; Vivancos *et al.*, 2005). It also has been reported that the chaperone function of *S. cerevisiae* Tsa1 requires the peroxidatic but not the resolving Cys of this Prx (Jang *et al.*, 2004). We can discount the participation of Tpx1 in heat-shock adaptation, because the growth of $\Delta tpx1$ cells at 37°C under anaerobic conditions was identical to that of a wild-type strain (data not shown). We show here that Tpx1.C169S is able to support aerobic growth, providing the first evidence of *in vivo* peroxidase activity of a 2-Cys Prx lacking the resolving Cys. As shown for other family members, Tpx1.C169S is also able to detoxify peroxides *in vitro*. Nevertheless, Tpx1.C169S is not fully functional, as shown by the impaired survival of a strain expressing this protein when exposed to mild extracellular H₂O₂ stress. Whether this limited peroxidase activity is a consequence of a decreased catalytic efficiency, an increased sensitivity to inactivation by peroxides, or both is yet to be determined.

Several H₂O₂ scavengers contain only one conserved Cys residue; for example, this is the case with mammalian GSH peroxidase, NADH peroxidase, and members of the 1-Cys Prx family (Kang *et al.*, 1998a; Poole *et al.*, 2004; Rhee *et al.*, 2005b). These proteins catalyze the reduction of peroxides, although the identity of the electron donor to reduce and recycle the Cys-SeOH or Cys-SOH to selenothiol or thiol groups, respectively, is still unknown for some of these H₂O₂ scavengers. We have demonstrated here that small thiols, such as DTT or GSH, can support the peroxidase activity of Tpx1.C169S *in vitro*, probably because these molecules can donate thiol groups to form disulfide linkages with the peroxidatic Cys of the other subunit (Tpx1.C48-SOH), and they can subsequently reduce the resultant disulfides. In our experiments, Tpx1.C169S also supports peroxidase activity via the Trx1 system. Poole and coworkers showed that the resolving Cys of the Prx AhpC is not essential for *in vitro* peroxidase activity when an excess of the reducing enzyme, AhpF, is supplied (Ellis and Poole, 1997). Even though it is unlikely that Trx1 provides the reducing capacity for the activity of Tpx1.C169S *in vivo*, because its concentration is normally 1000-fold lower than that of GSH (Danon, 2002), specific Tpx1–Trx1 protein interactions could mediate a more efficient reaction with this reducing system. Therefore, whether the intracellular GSH pool constitutes the *in vivo* thiol donor for Tpx1.C169S activity will need further investigation. The results presented here provide the first evidence that a 2-Cys Prx, Tpx1, does not require the resolving Cys for its peroxidase activity *in vivo*, and we propose that Tpx1.C169S can function as a 1-Cys Prx both *in vitro* and *in vivo*.

It has previously been reported that Tpx1 can be reversibly substrate-inactivated via the formation of a sulfinic acid on its peroxidatic Cys (Bozonet *et al.*, 2005; Vivancos *et al.*, 2005). We have shown in this report that Tpx1 is inactivated by high H₂O₂ doses *in vitro* and that this inactivation occurs concomitantly with the formation of Cys-48-SO₂H in Tpx1. Our *in vivo* and *in vitro* data indicate that oxidation of Cys-48-SH to sulfinic acid occurs when Tpx1 exists as a covalent dimer, before the accumulation of the inactive Tpx1 monomer. Rhee and coworkers proposed that during H₂O₂ detoxification the major covalent dimer formed in Prx en-

zymes contains two disulfide bonds, but that several minor dimeric forms carrying only one disulfide are also likely to be formed, resulting in a variable compactness and different mobilities on nonreducing SDS-PAGE (Chae *et al.*, 1994c). The sulfinylated dimer in our experiments migrates slower than the major, two disulfide-containing dimer, and it has the same electrophoretic mobility as the minor species carrying only one disulfide (data not shown). Therefore, only the minor band corresponding to the one disulfide-containing Tpx1 dimer undergoes sulfinylation. According to structural studies on Prxs, the peroxidatic and resolving Cys residues are situated far apart in the corresponding Prx dimers (Wood *et al.*, 2003b), and formation of a disulfide probably requires and induces significant conformational changes. Perhaps formation of the first disulfide in a Tpx1 dimer temporarily prevents the second Cys-48-SOH from stabilizing within its structural pocket in the active site, increasing its susceptibility to hyperoxidation to Cys-SO₂H.

ACKNOWLEDGMENTS

We thank Mercè Carmona for excellent technical assistance and José Ayté and members of the laboratory for very helpful discussions. We very much appreciate the advice from Elisa Cabiscol regarding protein carbonylation measurement as well as Michel Toledano for helpful reading of the manuscript. We also appreciate the generosity of Kaoru Takegawa (Kagawa University, Kagawa, Japan) for strain *cys1aΔ* and of Xavi Gomis-Ruth (Institut de Biologia Molecular de Barcelona, CSIC, Barcelona, Spain) for the pGEX-2T-TEV plasmid. This work was supported by Dirección General de Investigación, Spain, grants BMC2003-00220 and BFU2006-02610, and “Distinció de la Generalitat de Catalunya per a la Promoció de la Recerca Universitaria. Joves Investigadors” DURSI (Generalitat de Catalunya) to E.H.

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