

# Characterization of a Putative Thioredoxin Peroxidase Prx1 of *Candida albicans*

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In this study, we characterized a putative peroxidase Prx1 of *Candida albicans* by: 1) demonstrating the thioredoxin-linked peroxidase activity with purified proteins, 2) examining the sensitivity to several oxidants and the accumulation of intracellular reactive oxygen species with a null mutant (*prx1Δ*), a mutant (C69S) with a point mutation at Cys69, and a revertant, and 3) subcellular localization. Enzymatic assays showed that Prx1 is a thioredoxin-linked peroxidase which reduces both hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and *tert*-butyl hydroperoxide (*t*-BOOH). Compared with two other strong H<sub>2</sub>O<sub>2</sub> scavenger mutants for *TSA1* and *CAT1*, *prx1Δ* and C69S were less sensitive to H<sub>2</sub>O<sub>2</sub>, menadione and diamide at all concentrations tested, but were more sensitive to low concentration of *t*-BOOH. Intracellular reactive oxygen species accumulated in *prx1Δ* and C69S cells treated with *t*-BOOH but not H<sub>2</sub>O<sub>2</sub>. These results suggest that peroxidase activity of Prx1 is specified to *t*-BOOH in cells. In both biochemical and physiological cases, the evolutionarily conserved Cys69 was found to be essential for the function. Immunocytochemical staining revealed Prx1 is localized in the cytosol of yeast cells, but is translocated to the nucleus during the hyphal transition, though the significances of this observation are unclear. Our data suggest that *PRX1* has a thioredoxin peroxidase activity reducing both *t*-BOOH and H<sub>2</sub>O<sub>2</sub>, but its cellular function is specified to *t*-BOOH.

## INTRODUCTION

Prxs (peroxiredoxins), a large family of thiol-specific antioxidant proteins including thioredoxin peroxidases and alkyl hydroperoxide reductases, are observed across all kingdoms (Kang et al., 2005; Wood et al., 2003) six Prxs (PrxI–VI) were identified in mammalian cells (Lim et al., 1994; Rhee et al., 2001; Seo et al., 2000) and five Prxs (*TSA1*/cTPxI, *TSA2*/cTPxII, *AHP1*/cTPxIII, *PRX1*/mTPx, *DOT5*/nTPx) in *Saccharomyces cerevisiae* (Park et al., 2000; Wong et al., 2004). Prxs exert their protective antioxidant role in a cell through their peroxidase activity (ROOH + 2e<sup>-</sup> + 2H<sup>+</sup> → ROH + H<sub>2</sub>O), whereby hydrogen peroxide and a wide range of organic hydroperoxides (ROOH) are reduced

and detoxified (Rhee et al., 2001) with the use of electrons provided by thioredoxin (Trx) (Bryk et al., 2000; Hofmann et al., 2002; Jacobson et al., 1989; Peshenko and Shichi, 2001). The activity of Prxs is dependent upon an absolutely conserved peroxidatic cysteine that is essential for the hydroperoxide reduction step (Choi et al., 1998; Ellis and Poole, 1997). The Prx superfamily can be divided into three sub-groups, typical 2-Cys Prx, atypical 2-Cys Prx and 1-Cys Prx according to the number of conserved cysteinyl residues directly involved in catalysis (Chae et al., 1994a; 1999).

*Candida albicans*, the most prevalent human fungal pathogen, can cause diverse forms of candidiasis through systemic or life-threatening infections, especially in immunocompromised patients and reveals its pathogenicity through adhesion, morphological transition and secretion of proteases during evasion from the host immune system (Calderone and Fonzi, 2001). *C. albicans*, like other organisms, is suspected to have a variety of antioxidant enzymes, such as catalase, superoxide dismutase, glutathione peroxidase, thiol-specific antioxidant 1 (*TSA1*), to respond to the stress. No report on the characterization of Prx of *C. albicans* prompted us to identify a gene homologous with the *PRX1* gene of *S. cerevisiae*. The identified gene, *CaPRX1*, was found to encode a putative thioredoxin peroxidase, which is localized in the cytoplasm in yeast cells, while is translocated to the nucleus in hyphae. Hereafter, the two-letter prefix 'Ca' or 'Ca' is no longer used for genes or their protein products originated from *C. albicans*, while used for other species, for example, 'Sc' or 'Sc' for *S. cerevisiae*.

## MATERIALS AND METHODS

### Strains and cultures

*C. albicans* strains used in this study are described in Table 1. Cells were routinely grown at 30°C in liquid YPD medium (1% yeast extract, 2% peptone and 2% glucose). For hyphal induction, yeast cells grown to mid-log phase at 30°C were transferred to RPMI-1640 medium (Gibco-BRL, USA) or YPD supplemented with 10% fetal bovine serum (FBS) (Welgene, Korea) and further grown at 37°C for 2 h or at 37°C for 8 h to obtain germ tube or hyphal culture, respectively. The wild-type (wt) strain was SC5314 (Fonzi and Irwin, 1993). CAI4 was used

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Received November 15, 2011; revised December 19, 2011; accepted December 19, 2011; published online March 2, 2012

**Keywords:** hydrogen peroxide, peroxidase, *PRX1*, *tert*-butyl hydroperoxide, translocation

**Table 1.** Strains used in this study

Strain	Genotype	Source
SC5314	Wild-type	Fonzi and Irwin (1993)
CAI4	<i>ura3Δ::imm434/ura3Δ::imm434</i>	Fonzi and Irwin (1993)
<i>tsa1Δ/ tsa1Δ</i>	<i>ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG tsa1Δ::HIS1/tsa1Δ::hisG tsa1Δ::ARG4/tsa1Δ::hisG-URA3-hisG</i>	Shin et al. (2005)
<i>cat1Δ/ cat1Δ</i>	<i>ura3Δ::imm434/ura3Δ::imm434 cat1Δ::dpl200/cat1Δ::URA3-dpl200</i>	Nasution et al. (2008)
CPD1	<i>ura3Δ::imm434/ura3Δ::imm434 prx1Δ::hisG-URA3-hisG/PRX1</i>	This work
CPD1-U	<i>ura3Δ::imm434/ura3Δ::imm434 prx1Δ::hisG/PRX1</i>	This work
CPD2	<i>ura3Δ::imm434/ura3Δ::imm434 prx1Δ::hisG/prx1Δ::URA3-dpl200</i>	This work
CPD2-U ( <i>prx1Δ</i> )	<i>ura3Δ::imm434/ura3Δ::imm434 prx1Δ::hisG/prx1Δ::dpl200</i>	This work
CPD2+U	<i>ura3Δ::imm434/ura3Δ::imm434 prx1Δ::hisG/prx1Δ::dpl200 iro1::URA3</i>	This work
PRX1-R (CPD2-R)	<i>ura3Δ::imm434/ura3Δ::imm434 prx1Δ::hisG/prx1Δ::dpl200 iro1::PRX1-URA3</i>	This work
C69S	<i>ura3Δ::imm434/ura3Δ::imm434 prx1Δ::hisG/prx1Δ::dpl200 iro1::PRX1<sub>C69S</sub>-URA3</i>	This work

as a parental strain to construct a *prx1* null mutant (*prx1Δ*). All *prx1* disruptants were selected on synthetic dextrose (SD) medium composed of 0.67% yeast nitrogen base without amino acids (Difco, USA), 2% glucose, 0.06% uracil drop-out mix and 2% agar if needed. Uracil (80 mg/L) was added to liquid and solid media when culturing uracil auxotrophs. *Escherichia coli* strain DH5α was used to amplify all plasmids.

#### Molecular methods

Plasmid preparation, poly(A)+ RNA preparation, cloning, and nucleic acid and protein blotting analysis were performed as previously described (Sambrook and Russell, 2001). The nucleotide sequences were confirmed, if necessary.

#### Cloning of PRX1

After a sequence with highest similarity to *ScPRX1* was searched through BLAST, DNA fragments containing the open reading frame (ORF) of *PRX1* or ORF plus flanking sequences (or alternatively full gene; FG for short) were amplified from the genomic DNA of SC5314 with primer pairs 5'-GAATTCG **ATGAGAGACAAAAACAAAC**-3' (the initiation codon in bold)/5'-GAATTC**TTACTTGTCTTCCTTTTCCA**-3' (the termination codon in bold), and 5'-GAATTCGAAGCTACGGAGAAGATT CG-3'/5'-GAATTCATCAGTTCAAAGAATAATT-3', and sub-cloned into vectors pGEM-T Easy (Promega, USA) and pCR 2.1-TOPO vector (Invitrogen, USA), respectively, yielding T-PRX1<sub>ORF</sub> and TOPO-PRX1<sub>FG</sub>.

#### Construction of PRX1-related strains

For determination of possible physiological and biochemical properties of Prx1, we constructed a strain in which *PRX1* was nullified, C69S mutant in which Cys<sup>69</sup> was replaced by serine, and a revertant. Since two alleles of *PRX1* were found to be present in the genome of *C. albicans* (designated 'orf19.5180' according to the Candida Genome Database), two sequential disruptions were necessary to obtain a *PRX1*-null mutant. The presence or absence of *PRX1* allele was confirmed by Southern, Northern, and Western blot analyses (Supplementary Fig. 1).

For the first disruption of *PRX1* allele, the 4.0 kb *hisG-URA3-hisG* (URA Blaster) cassette from *KpnI* digestion of p5922 (Fonzi and Irwin, 1993) was blunt-ended with Klenow and then ligated into T-PRX1<sub>ORF</sub> which was digested with *BsmBI* and blunt-ended with Klenow. The resulting plasmid was designated pJM. The *PRX1* disruption cassette was obtained by *AatII/BglI* digestion of pJM and was transformed into strain CAI4. URA<sup>+</sup>

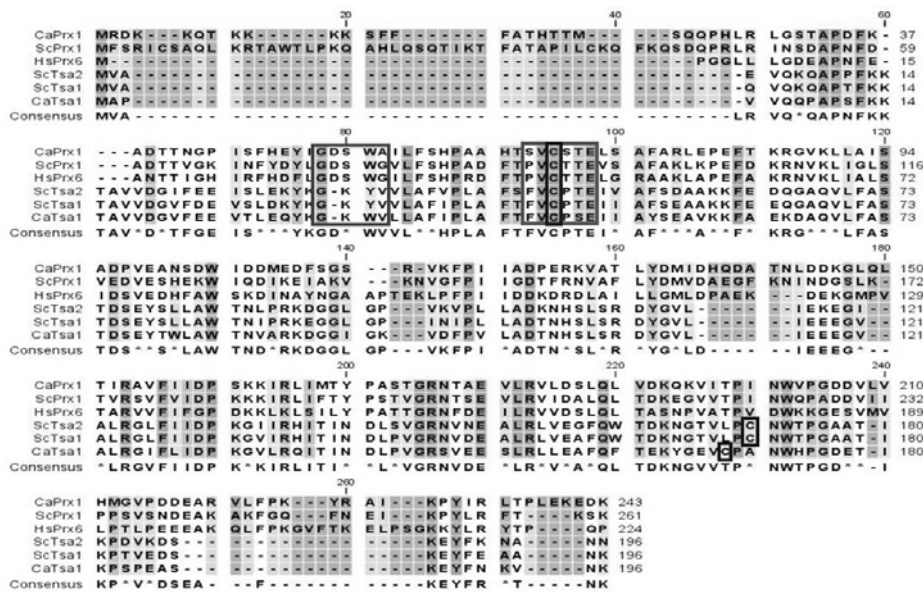
transformants were selected on a uracil-deficient medium to yield CPD1. For the second disruption of *PRX1* allele, CPD1-U was obtained by culturing CPD1 on SD medium containing 5-fluoroorotic acid (5-FOA; 625 mg/ml) and uridine (80 mg/L). Separately, the disruption cassette (*URA3-dpl200*) of pDDB57 (kindly provided by Dr. D. Davis) (Wilson et al., 2000) was amplified with primers containing 65 nucleotides of *PRX1* at the 5' end (sense, 5'-GACGTTCTCCCCCCTAGACCAATCGAAA GCCGTGGTATTATTCCGGGCTTTGAAGAAAAGTCTTTCT TTTTCCCAGTCACGACCTT-3'; antisense, 5'-CCTTAAACA ATAAAAAAAACGAGATCTTAAAAAATAAACTCTTCGTAA ATTTAAGGCACACGAAGTGGGTGGAATTGTGCGGATA-3'). CPD2, in which both alleles of *PRX1* are disrupted, was obtained by transformation of amplified *URA3-dpl200* cassette into CPD1-U and selection on a uracil-deficient medium.

We also constructed a strain complemented with *PRX1* by using *URA3* as a selection marker, *URA3* was again excised from the genome of CPD2 by FOA selection as above, generating CPD2-U (or alternatively *prx1Δ*). Separately, the 1.9 kb *SpeI/HindIII* fragment of TOPO-PRX1<sub>FG</sub> was cloned into the *SpeI* and *HindIII* site of *URA3*-containing pLUX (kindly provided by Dr. W. Fonzi) to yield pLUX-PRX1. pLUX-PRX1 was linearized by *NheI* and transformed into CPD2-U to integrate the functional *PRX1* gene into the *IRO1* locus. One of colonies selected on a uracil-deficient medium was designated CPD2-R (or alternatively *PRX1*-R). A strain (CPD2+U) with the plain pLUX integrated into the *IRO1* locus was also constructed to use as a control for the effect of the *URA3* copy present in the *IRO1* locus in the case of CPD2-R.

To construct C69S, PCR-mediated site-directed mutagenesis was performed with complementary primers (5'- GCTGCCCC CACCAAGTGTGAGTAGCACCAGCTTTCTG-3' and 5'- CAG AAAGCTCGGTGCTACTCACACTGGTGTGGGCAGC-3'), which contain a 1-base mismatch (bold) that converts the codon for 69<sup>th</sup> residue cysteine to serine, and pLUX-PRX1 as a template, yielding pLUX-PRX1<sub>C69S</sub>. The integration into the *IRO1* locus followed the same procedures as done for CPD2-R.

#### Preparation of Prx1 proteins

To produce wild type (Prx1<sub>wt</sub>) and mutant proteins (Prx1<sub>C69S</sub>), the Prx1-encoding DNA fragments were PCR-amplified with primers 5'-CGC GCTAGC AGA GAC AAA AAA CAA ACA AAA AAA AAA AA-3' containing a *NheI* site (underlined) and 5'- GC CTCGAG TTA CTT GTC TTC CTT TTC CAA CGG-3' containing both an *XhoI* site (underlined) and the stop codon (bold)



**Fig. 1.** Amino acid sequence alignment. Amino acid sequences of *PRX1* and *TSA1* of *C. albicans*, three TPxs of *S. cerevisiae*, and mammalian PrxVI were aligned according to priority of higher similarity. Conserved Cys in blue box. Peroxidase motif (PVCTTE) in red box. Lipase motif (GDSWA) in green box.

and either T-PRX1<sub>ORF</sub> or pLUX-PRX1<sub>C69S</sub> for template. Purified PCR products were digested with *NheI* and *XhoI*, and cloned into the pET-28b vector. The constructed plasmids were transformed into *E. coli* BL21 (DE3) to express Prx1 proteins via IPTG induction. Recombinant proteins were purified using Ni-chelating Sepharose column after sonication as manufacturer's recommendation (Amersham Biosciences). The fractions containing His-Prx1 protein were pooled and dialyzed against 20 mM Tris-HCl (pH 7.6), which were subsequently used for either antibody production (with the case of Prx1<sub>wt</sub>) or enzyme assay (with both Prx1<sub>wt</sub> and Prx1<sub>C69S</sub>).

#### Determination of peroxidase activity of CaPrx1

Yeast thioredoxin reductase 1 and thioredoxin 1 were purchased from Ab Frontier. A peroxidase reaction was carried out in a 200  $\mu$ l reaction mixture containing 50 mM HEPES (pH 7.0), 5 mM DTT, and 0.1 mM H<sub>2</sub>O<sub>2</sub> or 0.1 mM t-BOOH in the absence or presence of 2  $\mu$ M CaPrx1 at room temperature. The glutathione-dependent peroxidase activity was assayed with the reaction mixture containing glutathione instead of DTT. At the appropriate time, 5  $\mu$ l of the reaction mixture was added to 0.2 ml of FOX1 reagent, incubated at room temperature for 30 min, and then the remaining amount of peroxide was monitored by measuring the absorbance at 560 nm and calculated from the standard curve (Rhee et al., 2010). The FOX1 reagent contained 100  $\mu$ M xylenol orange, 250  $\mu$ M ferrous ammonium sulfate, 100 mM sorbitol, and 25 mM H<sub>2</sub>SO<sub>4</sub>; the iron salt was dissolved directly in acid, since ferrous ions are prone to autoxidation at physiological pH (Rhee et al., 2010).

#### Spot assay

Aliquots (10  $\mu$ l) containing approximately 10<sup>3</sup> cells of an overnight culture were spotted on YPD plates containing oxidants at indicated concentrations. Plates were observed after 2-3 days of incubation at 30°C (Lee et al., 1999).

#### Intracellular ROS assay

The relative amount of intracellular ROS was measured using CM-H<sub>2</sub>DCFDA (5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester) (Molecular Probe, USA) as

described previously (Bae et al., 1997).

#### Anti-CaPrx1 antibody production

The CaPrx1 ORF was cloned into pET-30b (Novagen, USA) and transformed into *E. coli* strain BL21 (DE3). Fusion proteins were purified according to the manufacturer and used for anti-CaPrx1 antiserum in the mouse.

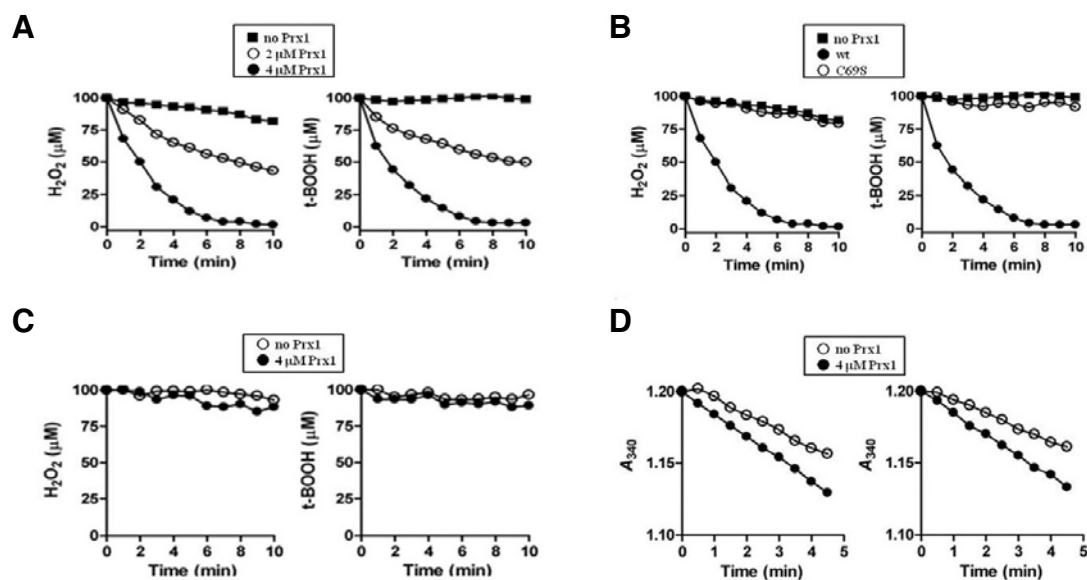
#### Subcellular localization of Prx1

Immunostaining was performed as described previously (Shin et al., 2005), with using anti-Prx1 antiserum and rhodamine-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, USA). Nuclei were counterstained by DAPI. Fluorescent images were captured with a LSM510 Meta confocal microscope (Carl Zeiss, Germany) at excitation 545 nm and emission 560 nm for rhodamine and at excitation 364 nm and emission BP 385-470 nm for DAPI.

## RESULTS

#### Amino acid sequence analysis of PRX1

When Blast was performed from the Candida Genome Database [Stanford Genome Technology Center (<http://sequence-www.stanford.edu/group/candida>)] with the amino acid sequence of ScPRX1 (YBL064C) as a query, PRX1 (GenBank accession number: XP\_717002) was found to have highest similarity (55%) among *C. albicans* homologs (Fig. 1). The deduced amino acid sequence revealed that Prx1 has only one conserved cysteine (Cys69), corresponding to Cys47 of ScTsa1, thus, it can be classified as a member of 1-Cys Prx family. In fact, Prx1 showed around 30% similarity with 2-Cys Prx members Tsa1 and ScTsa1 (Pedrajas et al., 2000), but 46% similarity with phylogenically distant human PrxVI, a 1-Cys Prx member (Chae et al., 1994b) (Fig. 1). Interestingly, Prx1 has no organelle-targeted sequence other than the nuclear localization signal (NLS) (<sup>8</sup>KKKK<sup>11</sup>) at the N-terminus, contrasting the mitochondrial localization signal of ScPrx1 (<sup>2</sup>FSRICSAQLKRT<sup>13</sup>) at the N-terminal region. In addition, the presence of a single putative lipase motif (<sup>52</sup>GDSWA<sup>56</sup>) represents Ca<sup>2+</sup>-independent phospholipase A<sub>2</sub> activity (Nevalainen, 2010), which functions



**Fig. 2.** Peroxidase activity of Prx1. Mean values of triplicate experiments are shown. (A) DTT-dependent removal of H<sub>2</sub>O<sub>2</sub> (left panel) and t-BOOH (right panel) by Prx1. Peroxidase reaction was carried out at room temperature in 200 μl of reaction mixture containing 50 mM HEPES (pH 7.0), 5 mM DTT, 100 μM H<sub>2</sub>O<sub>2</sub> or 100 μM t-BOOH, and in the absence (closed square) or presence of Prx1 (closed circle, 2 μM; closed triangle, 4 μM). At the indicated times, the remaining peroxides were measured using FOX1 reagent as described in Materials and methods. (B) Peroxidase reaction was carried as in (A) with 100 μM H<sub>2</sub>O<sub>2</sub> (left panel) or 100 μM t-BOOH (right panel) in the absence (closed square) or presence of 4 μM Prx1 (wild type, closed circle; C69S, closed triangle). (C) Peroxidase reaction was performed using 4 μM Prx1 as in (A), except the presence of GSH instead of DTT as a reducing equivalent: closed square, without Prx1; closed triangle, with Prx1. (D) Peroxidase activity linked to NADPH oxidation with *S. cerevisiae* Trx system was monitored by decreases of A<sub>340</sub> in a 200-μl reaction mixture containing 50 mM HEPES (pH 7.0), 0.2 mM NADPH, 2 μM ScTrr1, 5 μM ScTrx1, 4 μM Prx1, and either 0.1 mM H<sub>2</sub>O<sub>2</sub> (left panel) or 0.1 mM t-BOOH (right panel): closed square, without Prx1; closed triangle, with Prx1.

to protect cell membrane phospholipid against oxidative damage.

#### Thioredoxin peroxidase activity of Prx1

To test whether Prx1 has a peroxidase activity, Prx1<sub>wt</sub> and Prx1<sub>C69S</sub> were included in the reaction mixture containing DTT as a reducing equivalent and naturally occurring hydroperoxide H<sub>2</sub>O<sub>2</sub> or t-BOOH as a substrate. Prx1<sub>wt</sub> removed both H<sub>2</sub>O<sub>2</sub> and t-BOOH at a similar rate, which was dependent on Prx1 concentration (Fig. 2A), whereas Prx1<sub>C69S</sub> resulted in complete loss of the enzymatic activity (Fig. 2B). These data demonstrate that Prx1 is a thiol-linked peroxidase and that conserved Cys69 is essential for the peroxidase activity.

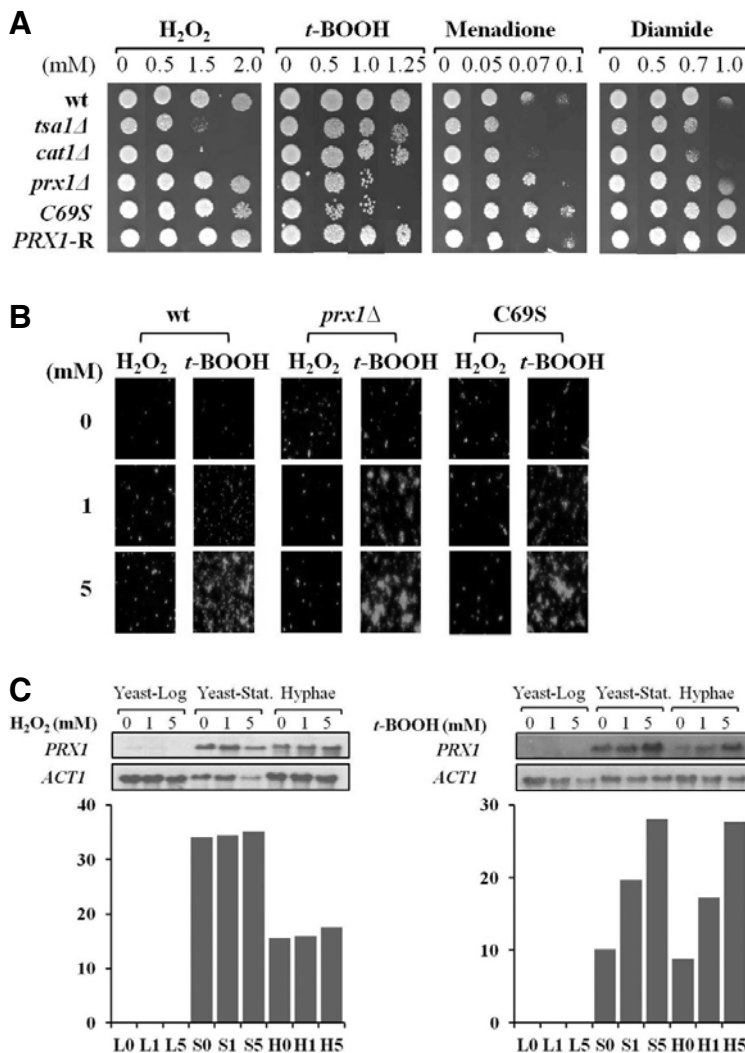
As an attempt to find a physiological reducing equivalent for Prx1, its peroxidase activity toward H<sub>2</sub>O<sub>2</sub> and t-BOOH was assessed in the presence of GSH or *S. cerevisiae* thioredoxin system, which includes NADPH, thioredoxin reductase and thioredoxin 1 of *S. cerevisiae* (Ab Frontier, USA). The peroxidase activity of Prx1 was barely supported by GSH (Fig. 2C), whereas the *S. cerevisiae* thioredoxin system marginally provided electrons to Prx1 (Fig. 2D). Prx1 is a thiol-dependent peroxidase and the results suggest that Prx1 is likely a thioredoxin-dependent peroxidase. However, further study is necessary to confirm this possibility.

#### Sensitivity to oxidants

After confirming that there was no position effect of the *URA3* copy of CPD2+U and CPD2-R (or *PRX1*-R) (data not shown), we examined the sensitivity of *PRX1*-related strains (*prx1Δ*, C69S, *PRX1*-R) to H<sub>2</sub>O<sub>2</sub>, t-BOOH, and two artificial oxidants diamide and menadione by exploiting spot assay. For compari-

son, wt and null mutants (*tsa1Δ* and *cat1Δ*) for known antioxidant genes *TSA1* and *CAT1*, respectively, were also used. As shown in Fig. 3A, *prx1Δ* and C69S were less sensitive to H<sub>2</sub>O<sub>2</sub>, menadione and diamide at all concentrations tested, but more sensitive to low concentration of t-BOOH than *tsa1Δ* and *cat1Δ* (Fig. 3A). Meanwhile, both *cat1Δ* and *tsa1Δ* were highly sensitive to menadione, suggesting a role in detoxification of H<sub>2</sub>O<sub>2</sub> formed by sequential reduction of superoxide anion generated by menadione (Jamieson, 1992). These data suggest that Prx1 is specialized in reducing t-BOOH but not H<sub>2</sub>O<sub>2</sub> *in vivo*. Additionally, the strain C69S exhibited a similar sensitivity pattern to that of *prx1Δ*, demonstrating that Cys69 is essential for the biological activity of Prx1.

The spot assay results were further evidenced by measuring intracellular ROS in wt, *prx1Δ*, and Cys69 cells treated with H<sub>2</sub>O<sub>2</sub> or t-BOOH with a fluorescent probe H<sub>2</sub>DCFDA, which increases intensity when oxidized to DCF by ROS. If Prx1 acts as an antioxidant and the presence of Cys69 is an important factor for the function, ROS might be accumulated in *prx1Δ* as well as in C69S mutant. Figure 3B shows that fluorescence intensity was significantly increased in *prx1Δ* and C69S compared with that in wild type strain under normal aerobic condition. However, when exogenous H<sub>2</sub>O<sub>2</sub> (1 mM) was challenged to the cells, the fluorescence level of mutants decreased to a similar level with wt and even below the level of wt under high concentration of H<sub>2</sub>O<sub>2</sub> (5 mM). When mutant cells were treated with t-BOOH, however, the fluorescence was dramatically increased to much more level than that of H<sub>2</sub>O<sub>2</sub> treatment, again indicating that Prx1 reduce t-BOOH but not H<sub>2</sub>O<sub>2</sub> and that Cys69 is an essential residue for Prx1 activity. The results of either the spot or ROS assays imply that the major biological



**Fig. 3.** Antioxidant activity of *PRX1*. (A) Spot assay. Ten-fold dilutions of SC5314 (wt), *tsa1Δ*, *cat1Δ*, *prx1Δ*, C69S, and *PRX1*-R cells were exposed to  $H_2O_2$ ,  $t$ -BOOH, diamide, and menadione on YPD plates at given concentrations. (B) Intracellular ROS assay. wt, *prx1Δ*, and C69S cells were treated with  $H_2O_2$  or  $t$ -BOOH for 1 h and assayed for intracellular ROS by fluorescence. (C) Northern analysis of *PRX1* in response to  $H_2O_2$  and  $t$ -BOOH. wt was cultured in YPD until mid-log phase and stationary phase. Hyphae were differentiated with 10% serum in YPD for 6 h. RNAs were prepared from cells treated with  $H_2O_2$  and  $t$ -BOOH at given concentrations for 1 h and subjected to Northern analysis.

substrate of Prx1 is  $t$ -BOOH not  $H_2O_2$ . The discrepancy between biochemical (Figs. 2A and 2B) and physiological data (Figs. 3A and 3B) is probably due to that the activity of Prx1 for removing  $H_2O_2$  was compensated by two strong  $H_2O_2$  scavenger Cat1 and Tsa1 in cells.

It was of our concern whether the *PRX1* transcript is influenced by the increased concentration of either  $H_2O_2$  or  $t$ -BOOH during the growth. When poly(A)<sup>+</sup> RNA samples prepared from exponentially growing yeast cells were subjected to Northern blot analysis, no messages were detected (Fig. 3C). So, we continued to examine the expression of *PRX1* in cells of stationary phase and hyphae. It was shown that *PRX1* was expressed relatively high by both  $H_2O_2$  and  $t$ -BOOH, but inducible in a dose-dependent manner by  $t$ -BOOH only (Fig. 3C). Although the biological significance of phase-specific expression of *PRX1* is unclear, these data show that cells at the stationary phase during yeast growth and the hyphal phase respond to increased  $t$ -BOOH but  $H_2O_2$  doesn't increase the RNA level.

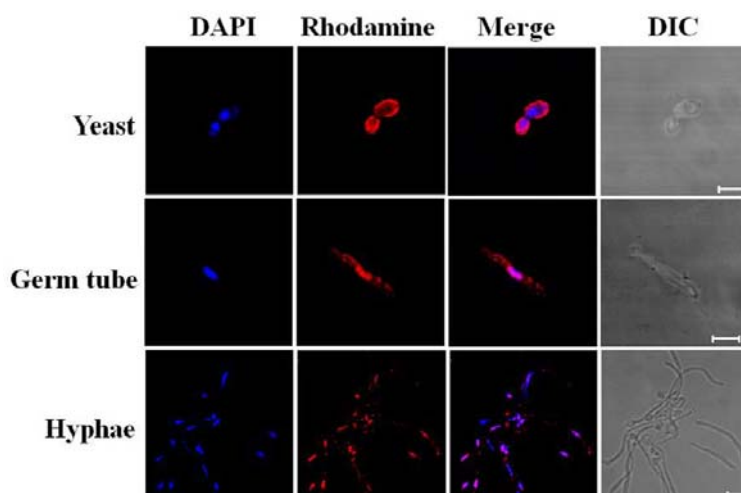
#### Sub-cellular localization of Prx1

Five isoforms of thioredoxin peroxidases of *S. cerevisiae* are classified according to their sub-cellular localizations; cTPxI, cTPxII, and cTPxIII in the cytoplasm, mTPx (also called ScPrx1)

in the mitochondria, and nTPx in the nucleus (Park et al., 2000). As mentioned earlier, Prx1 has a nuclear localization signal at the N-terminus instead of a mitochondria targeting sequence shown in ScPrx1. When immunocytochemical staining was performed with Prx1-specific antibody (Fig. 4), Prx1 was apparently localized in the cytoplasm (first row). However, it was translocated to the nucleus in both germ tubes and hyphal cells induced by 10% serum (second and third rows), suggesting that the localization of Prx1 is controlled by signal transduction. Thus, Prx1 behaves differently from ScPrx1 with highest amino acid similarity. The biological significance of nuclear translocation of Prx1 remains to be further clarified.

#### DISCUSSION

A search of the Candida Genome Database reveals six putative peroxidases including *AHP1*, *AHP2*, *DOT5*, *TRP99*, *TSA1*, and *orf19.5180* (*PRX1*). *TSA1* and *PRX1* are homologs of cTPxI and mTPx of *S. cerevisiae* respectively. *DOT5* is homologous with *ScDOT5*hTPx and *AHP1*, *AHP2*, and *TRP99* are homologous with *ScAHP1*/cTPxIII. Of those, only *TSA1* has been characterized as an antioxidant (Shin et al., 2005; Urban et al., 2005), while the rest have remained uncharacterized.



**Fig. 4.** Subcellular localization of Prx1. *In situ* hybridization with anti-Prx1 antibody was performed in yeast cells, germ tubes, and hyphae. Scale bars, 5  $\mu$ m.

From this study, *PRX1* becomes a second Prx member proven to have peroxidase activity in *C. albicans*. Using the pSORT II program (<http://www.psori.org/>), *DOT5*, *AHP1* and *AHP2*, and *TRP99* were localized to the nucleus, cytoplasm, and mitochondria, respectively. In this regard, *ScPRX1*/mTPx used for a BLAST query to search *PRX1* indicates a close relationship with *TRP99*. Thus, in *C. albicans*, it is highly possible that three major fractionated compartments (nucleus, cytoplasm, and mitochondria) possess their own Prxs.

The translocation of Prx1 from the cytosol to the nucleus in hyphal cells (Fig. 4) is an interesting observation. As typically exemplified by I- $\kappa$ B/NF- $\kappa$ B and steroid receptors, nuclear translocation of proteins is induced by extra/intracellular signals and mostly results in gene activation. It is unlikely that the nuclear translocation of Prx1 is simply aimed at reducing nuclear  $H_2O_2$ , since *DOT5* is predicted to be present in the nucleus. However, the doubling time of the *prx1* $\Delta$  was almost same as the wild-type, and no apparent physiological changes were observed under the hyphae-inducing condition (data not shown). The only effect of *PRX1* deletion was some sensitivity to oxidative stresses. Whether the nuclear translocation of Prx1 is a cause or a consequence of hyphal differentiation remains to be answered.

The *prx1* null mutant showed weak sensitivity toward  $H_2O_2$  and hypersensitivity toward *t*-BOOH (Fig. 2A), suggesting that Prx1 may act as a peroxidase for organic peroxides in cells. This was consistent with the results of the ROS assay (Fig. 2B). In contrast, the high sensitivity of *tsa1* $\Delta$  and *cat1* $\Delta$  to menadione and diamide suggests that Tsa1 and Cat1 are antioxidants for  $H_2O_2$  detoxification and also might be involved in the major stress responses, as diamide has pleiotropic effects and can elicit a wide range of cellular damage (Trotter and Grant, 2002). In contrast to the inducibility of *PRX1* expression with *t*-BOOH (Figs. 3A and 3B), however, recombinant Prx1 protein displayed peroxidase activity for both  $H_2O_2$  and *t*-BOOH with a similar rate (Fig. 3A). These data indicate that an unidentified regulation system may control the function of different antioxidants in *C. albicans*. ScYap1, a bZIP transcription factor of *S. cerevisiae*, regulates the cellular response to oxidative stress, and Cap1 of *C. albicans* is a functional homologue with ScYap1 (Zhang et al., 2000). In the upstream region of the *PRX1* ORF there are two Cap1-binding sequence: TTAATA (Zhang et al., 2000) and TTAGTGA (Pedrajas et al., 2000) at -430 and -319 from the start codon, respectively. Furthermore, seven stress

response elements of a consensus sequence AGGGG are also found. Thus, *PRX1* could respond to a range of stresses through interaction with different transcription factors and genes. A detailed analysis for the cell protective function and molecular mechanism such as redox cycle of Prx1 is needed.

*Note: Supplementary information is available on the Molecules and Cells website ([www.molcells.org](http://www.molcells.org)).*

#### ACKNOWLEDGMENTS

This work was supported by Korea Science and Engineering Foundation grant funded by the Korea government (Ministry of Science and Technology) (No. 2006-0063-2), the Pioneer Research Center Program (No. 2009-0081512) and Bio R&D Program Grant M10642040002-07N4204-00210 (to W.J.) through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology. K.S. and J.K. were recipients of Ewha Global Partnership Program 2006 and the Brain Korea 21 Scholars Program, respectively.

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