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Mitochondrial and cytosolic expression of human peroxiredoxin 5 in *Saccharomyces cerevisiae* protect yeast cells from oxidative stress induced by paraquat

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Abstract Human peroxiredoxin 5 is a recently discovered mitochondrial, peroxisomal and cytosolic thioredoxin peroxidase able to reduce hydrogen peroxide and alkyl hydroperoxides. To gain insight into peroxiredoxin 5 antioxidant role in cell protection, we investigated the resistance of yeast cells expressing human peroxiredoxin 5 in mitochondria or in the cytosol against oxidative stress induced by paraquat. The herbicide paraquat is a redox active drug known to generate superoxide anions in mitochondria and the cytosol of yeast and mammalian cells leading to the formation of several reactive oxygen species. Here, we report that mitochondrial and cytosolic human peroxiredoxin 5 protect yeast cells from cytotoxicity and lipid peroxidation induced by paraquat.

Key words: Peroxiredoxin; Paraquat; Yeast; Mitochondria; Cytosol; Oxidative stress

1. Introduction

Peroxiredoxins (PRDXs) are enzymes that play an important role in eukaryotes and prokaryotes in reducing hydrogen peroxide (H_2O_2), organic hydroperoxides (ROOH) and peroxynitrite ($ONOO^-$) by the use of reducing equivalents derived from thiol-containing donor molecules such as thioredoxin, glutathione, tryparedoxin, alkyl hydroperoxide reductase subunit F (AhpF) and cyclophilin A [1–5]. Six PRDXs have been discovered in mammals with distinct subcellular localizations and a wide tissue distribution. Mammalian PRDXs have been implicated in the modulation of signaling cascades leading to cell differentiation, proliferation and immune response but also in direct antioxidant protection (for review see [6–10]).

Peroxiredoxin 5 (PRDX5), also known as PrxV, AOEB166, PMP20 or ACR1, is a mammalian thioredoxin peroxidase that can be addressed to mitochondria, peroxisomes and the cytosol [11–14]. PRDX5 ortholog in *Saccharomyces cerevisiae* is alkyl hydroperoxide reductase 1 (Ahp1p) also named Ylr109w, PMP20 and cTpxIII [15,16]. Moreover, Ahp1p is addressed in yeast to peroxisomes and the cytosol [17].

Paraquat is a redox active drug and its toxic effect is thought to be mediated by reactive oxygen species (ROS) produced by the enzymatic one-electron reduction of paraquat at the expense of NADPH or NADH, followed by one-electron transfer to dioxygen (O_2), generating superoxide anion ($O_2^{\bullet -}$) [18]. These reactions are catalyzed in mitochondria by the outer membrane NADH–cytochrome b5 oxidoreductase and by the inner membrane NADH–ubiquinone oxidoreductase [19]. Moreover, paraquat is also reduced by the endoplasmic reticulum NADPH–cytochrome P450 oxidoreductase [19]. ROS generated by paraquat can damage a wide variety of cellular constituents including DNA, RNA, proteins, sugars and lipids and thereby compromise cell viability [20]. Mitochondria are a main source of ROS and an important target for oxidative injury. Also, H_2O_2 induces mitochondrial permeability transition and disrupts the mitochondrial membrane potential. Such conditions in mammalian cells can lead to the release of cytochrome *c* from the mitochondria to the cytosol and can trigger apoptosis by activating pro-caspase 9 [21].

To examine the role of human PRDX5 in the cellular defense against paraquat toxicity, PRDX5 was expressed in the cytosol and mitochondria of *S. cerevisiae*. The effects of human PRDX5 expression on paraquat-induced cytotoxicity in yeast were then examined and revealed that mitochondrial and cytosolic PRDX5 protect yeast cells from oxidative stress induced by paraquat.

2. Materials and methods

2.1. Strains, media and growth conditions

S. cerevisiae strains used in this study were previously described [22]. *ahp1*Δ (*leu2-3,112 his3-1 ura3-52 trp1-289 ahp1*Δ*::KAN*^{R}) and its corresponding wild-type (WT) (*leu2-3,112 his3-1 ura3-52 trp1-289*) were the meiotic segregants obtained by sporulation of CEN. AHP1/*ahp1* [22]. The haploid WT strain was used to prepare genomic DNA for polymerase chain reaction (PCR) amplification of *AHP1* promoter. Yeast cells were grown aerobically at 30°C in standard YPD or SD-URA medium with 2% glucose and amino acids.

2.2. DNA techniques

Standard protocols for nucleic acid manipulation and yeast transformation were used [23]. All DNA constructs were sequenced with the ABI Prism Big Dye Terminator Cycle Reaction Kit based on the dideoxy chain termination method (Applied Biosystems).

2.3. Construction of plasmids

The human *PRDX5* cDNA (GenBank accession number NM_012094) coding for the cytosolic form of PRDX5 without the

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Abbreviations: PRDX5, peroxiredoxin 5; ROS, reactive oxygen species; PCR, polymerase chain reaction; WT, wild-type; COX4, cytochrome *c* oxidase subunit 4

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mitochondrial presequence [11] under the control of the yeast *AHP1* promoter (GenBank accession number Z73281) was obtained by PCR-mediated overlap extension [24] and cloned into vector YEp24 [25]. The initial two PCRs were performed with primers 5′-AHP1-BamHI (5′-GTTGTTGGATCCCTTTGGATATTCGTGTTCAAG-3′) and 3′-AHP1-PRDX5 (5′-CACCTTGATTGGGGCCATGGC|-GTTTTGTTGTGGTTATTGGTAG-3′) for *AHP1* promoter amplification and 5′-AHP1-PRDX5 (5′-CTACCAATAACCACAACAAA-AC|GCCATGGCCCCAATCAAGGTG-3′) and 3′-PRDX5-SalI (5′-AATCAGGTCGACCCGCAAACCACAAGTGTTTATTG-3′) for *PRDX5* amplification. Underlined codons are *Bam*HI and *Sal*I sites. The switch between *AHP1* promoter and *PRDX5* cDNA is indicated (|). The two PCR products, which had a common 43 bp overlap were used as templates in a PCR-mediated overlap extension with primers 5′-AHP1-BamHI and 3′-PRDX5-SalI to create the amplicon. The p*PRDX5* plasmid was obtained by cloning the resulting 1226 bp PCR product into the *Bam*HI–*Sal*I sites of the vector YEp24 (Fig. 1A).

The same strategy was used as described above to construct the human *PRDX5* cDNA with the mitochondrial presequence under the control of the yeast *AHP1* promoter. Primers 5′-AHP1-PRDX5 and 3′-AHP1-PRDX5 were replaced by primers 5′-AHP1-MITO-PRDX5 (5′-CTACCAATAACCACAACAAAAC|GGTATGGGA-CTAGCTGGCGTG-3′) and 3′-AHP1-MITO-PRDX5 (5′-CACGC-CAGCTAGTCCCATACC|GTTTTGTTGTGGTTATTGGTAG-3′), respectively. The switch between *AHP1* promoter and *MITO-PRDX5* cDNA is indicated (|). The p*MITO-PRDX5* plasmid was obtained by cloning the resulting 1342 bp PCR product into the *Bam*HI–*Sal*I sites of the vector YEp24 (Fig. 1B).

The human *PRDX5* cDNA in fusion with the sequence coding for the mitochondrial presequence of yeast cytochrome *c* oxidase subunit 4 (COX4) (GenBank accession number Z72709) under the control of the yeast *AHP1* promoter was obtained by PCR-mediated overlap extension and cloned into vector YEp24. The initial three PCRs were performed with primers 5′-AHP1-BamHI and 3′-AHP1-COX4 (5′-GATTGACGTAGTGAAAGCAT|GTTTTGTTGTGGTTATTG-GTAG-3′) for *AHP1* promoter amplification, 5′-AHP1-COX4 (5′-CTACCAATAACCACAACAAAAC|ATGCTTTCACTACGTCAA-TC-3′) and 3′-MITO-COX4-PRDX5 (5′-CTCCCACCTTGATTGG-GGC||AAGCAGATATCTAGAGCTACAC-3′) for *MITO-COX4* amplification and 5′-MITO-COX4-PRDX5 (5′-GTGTAGCTCT-AGATATCTGCTT||GCCCCAATCAAGGTGGGAG-3′) and 3′-PRDX5-SalI for *PRDX5* amplification, respectively. The switch between *AHP1* promoter and *COX4* mitochondrial targeting sequence is indicated (|) as well as between *COX4* mitochondrial targeting sequence and *PRDX5* cDNA (||). The two first PCR products, which had a common 42 bp overlap were used as templates in a fourth PCR with primers 5′-AHP1-BamHI and 3′-MITO-COX4-PRDX5 to link *AHP1* and *COX4* together. The third and fourth PCR products which had a common 41 bp overlap were used as templates in a PCR-mediated overlap extension with primer 5′-AHP1-BamHI and 3′-PRDX5-SalI to create the amplicon. The p*MITO-COX4-PRDX5* plasmid was obtained by cloning the resulting 1295 bp PCR product into the *Bam*HI–*Sal*I sites of the vector YEp24 (Fig. 1C).

2.4. Yeast protein extraction and Western blotting

Yeast protein extraction was performed as previously described [26]. Protein concentration was determined by the BCA protein assay (Pierce). Proteins were separated on a standard 12% polyacrylamide gel, under reducing conditions [23]. Gels were subsequently electroblotted onto a nitrocellulose membrane. Rabbit polyclonal anti-PRDX5 (1:1000) [27], goat anti-rabbit horseradish peroxidase conjugate (1:6000) (Dako), and ECL Western blotting detection reagent (Amersham Pharmacia Biotech) were used. Kodak 1D Image Analysis Software (Eastman Kodak Company) was used for band quantification. Comparison of band intensities of PRDX5 in the different yeast strains with those of various amounts of recombinant 6×His-PRDX5 [28] allowed to estimate the amounts of PRDX5 in ng/μg total soluble protein.

2.5. Fluorescence microscopy

Red MitoTracker (Molecular Probes) was used to stain yeast mitochondria. Briefly, cells were incubated in a buffer solution (5% glucose, 10 mM HEPES, 0.5 μM Red MitoTracker) for 30 min at 30°C and then washed three times with phosphate-buffered saline (PBS). Cells were fixed with 4% paraformaldehyde in PBS for 1 h in microcentrifuge tubes, then washed three times with solution A (1.2 M sorbitol, 50 mM KPO_4, pH 7.0) and resuspended in 500 μl solution B (solution A, 0.14 M β-mercaptoethanol, 0.6 mg/ml of zymolase 100 000 T (Sigma) and 0.02% of glusulase (NEN)). After incubation at 37°C for 45 min, cells were washed twice with PBS, once with PBS containing 0.1% Triton X-100 and immersed 30 min in Tris-buffered saline (0.05 M, pH 7.6) (TBS) with 0.1% Triton X-100 (TBS-T) and 10% non-fat milk. Cells were incubated overnight at 4°C in TBS-T containing 1% non-fat milk and 1:200 rabbit polyclonal anti-PRDX5. Cells were washed three times with TBS-T and incubated with TBS-T containing 1:50 donkey anti-rabbit secondary antibody (Jackson) coupled to FITC for 1 h. Finally, cells were washed twice with TBS-T and once with TBS. Cells were incubated for 1 h at room temperature. Finally, cells were mounted in Mowiol with DAPI (50 μg/ml) and examined by fluorescence microscopy.

2.6. Phenotypical analyses

For toxicity assay on solid media, aliquots (3 μl) from overnight cultures in SD-URA medium containing 20×10^3 cells were spotted on YPD plates containing paraquat (Sigma) at different concentrations. Plates were monitored after 3 days of incubation at 30°C.

2.7. Measurement of lipid peroxidation

Lipid peroxidation was quantified by determination of thiobarbituric acid-reactive substances (TBARS) as previously described [22].

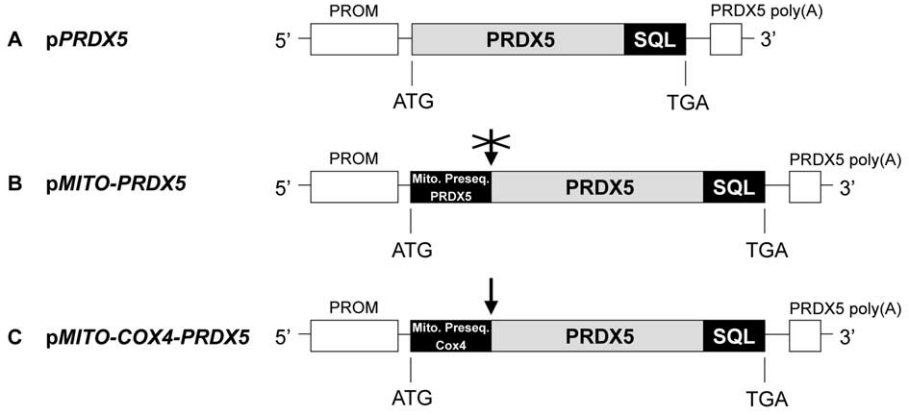

Fig. 1. Structure of the constructs cloned in yeast expression vector YEp24. A: p*PRDX5*; B: p*MITO-PRDX5*; C: p*MITO-COX4-PRDX5*. ATG, initiation codon; TGA, termination codon; PRDX5-poly(A), PRDX5 polyadenylation signal; SQL, PRDX5 peroxisomal targeting sequence type 1 Ser-Gln-Leu; Mito. Preseq. PRDX5, human mitochondrial presequence of PRDX5; Mito. Preseq. COX4, yeast mitochondrial presequence of PRDX5; PROM, promoter of yeast *AHP1* gene. The barred arrow in B indicates the non-cleavage of the human mitochondrial presequence in *S. cerevisiae* whereas the normal arrow in C indicates the cleavage of the yeast COX4 mitochondrial presequence in *S. cerevisiae* (see also Fig. 2).

2.8. Statistics

The data were analyzed by one-way analysis of variance and the significant differences between the controls and the treated groups were evaluated by post-hoc Newman–Keuls test. A P value <0.05 was considered significant.

3. Results

3.1. Expression and subcellular localization of human PRDX5 in yeast

WT yeast transformed with plasmids p*PRDX5* (coding for the short cytosolic and peroxisomal PRDX5), p*MITO-PRDX5* (coding for the long mitochondrial PRDX5), or p*MITO-COX4-PRDX5* (coding for the short PRDX5 in fusion with *S. cerevisiae* COX4 mitochondrial targeting sequence) (Fig. 1) were assayed for PRDX5 expression by immunoblotting (Fig. 2) and immunofluorescence microscopy (Fig. 3). Fig. 2 shows a band of 17 kDa corresponding to PRDX5 (lane 1), a band of 22 kDa corresponding to uncleaved MITO-PRDX5 (lane 2), and two bands of 17 kDa and 22 kDa corresponding, respectively, to cleaved and uncleaved MITO-COX4-PRDX5 (lane 3). WT yeast and *ahp1*Δ mutant transformed with the empty vector YEp24 did not show any immunoreactivity (Fig. 2, lanes 4 and 5). Different amounts of the 18 kDa recombinant 6×His-PRDX5 were also loaded in the gel (Fig. 2, lanes 6–8) [28]. The estimated amounts of PRDX5 in yeast strains are indicated below the blot (Fig. 2).

Immunofluorescence microscopy with the anti-PRDX5 antibody [27] in yeast revealed that PRDX5 and MITO-PRDX5 had a cytosolic localization (Fig. 3A). Human peroxisomal targeting sequence Ser-Gln-Leu (SQL) and human mitochondrial targeting sequence of PRDX5 were not functional in yeast (Fig. 3A). The localization of MITO-COX4-PRDX5 in yeast showed a reticular pattern which was proven to be mitochondrial as demonstrated by the staining of these organelles by Red MitoTracker (Fig. 3B). No immunoreactivity with anti-PRDX5 antibody was detected in WT yeast and *ahp1*Δ mutant transformed with the empty vector YEp24 (Fig. 3A).

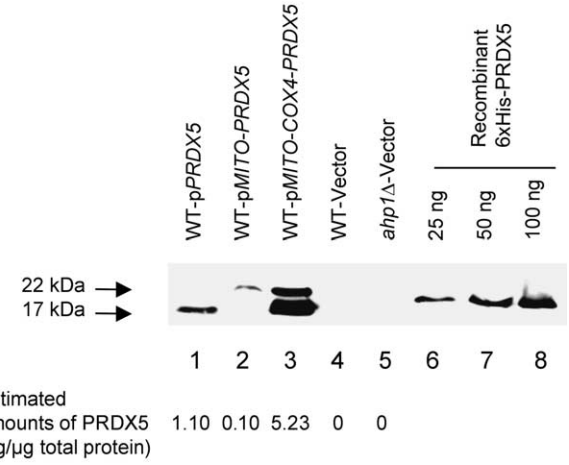

Fig. 2. Expression of human PRDX5 in yeast. Forty μg of total soluble protein from cellular extracts from WT-p*PRDX5*, WT-p*MITO-COX4-PRDX5*, WT-Vector and *ahp1*Δ-Vector, 120 μg of protein from cellular extracts from WT-p*MITO-PRDX5* and recombinant 6×His-PRDX5 were loaded in 12% SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis), followed by Western blotting and immunodetection with polyclonal rabbit anti-PRDX5. The lanes 6, 7 and 8 contain the indicated amounts of recombinant 6×His-PRDX5. Comparison of band intensities of PRDX5 in the different yeast strains with those of various amounts of recombinant 6×His-PRDX5 allowed to estimate the amounts of PRDX5 in ng/μg total soluble protein.

3.2. Lipid peroxidation in yeast

Lipid peroxidation can be followed by measuring in the medium the levels of malondialdehyde (MDA), which is mainly produced by decomposition of lipid hydroperoxides caused by ROS. To determine whether paraquat can cause lipid peroxidation and whether PRDX5 is able to protect cells from lipid peroxidation, the amounts of MDA produced by yeast cells cultured in liquid SD-URA containing paraquat were measured by the TBARS method. Induction of lipid peroxidation with 5 and 10 mM paraquat was observed in WT-p*MITO-PRDX5*, WT-Vector and *ahp1*Δ-Vector. At 5 mM paraquat, lipid peroxidation in WT-p*PRDX5* and WT-p*MITO-COX4-PRDX5* was significantly lower compared to WT-Vector (Fig. 4). At 10 mM paraquat, only WT-p*MITO-COX4-PRDX5* showed significant lower lipid peroxidation compared to WT-Vector (Fig. 4).

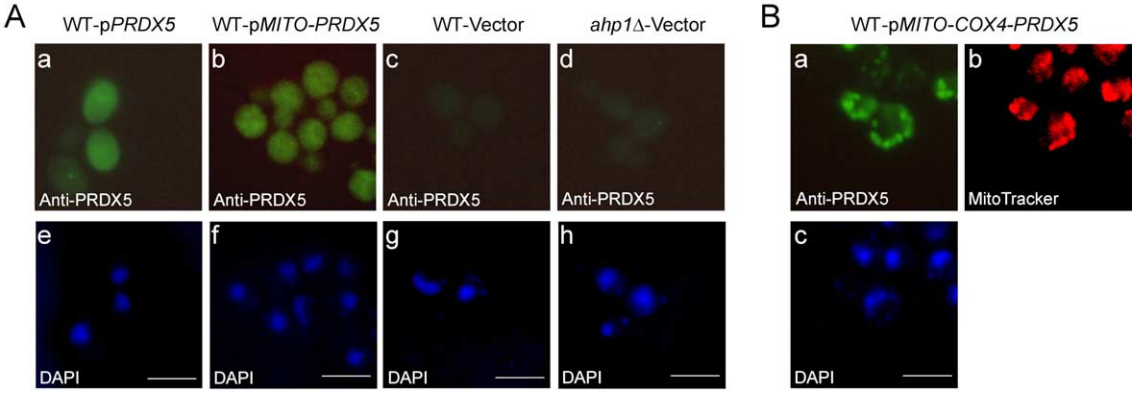

Fig. 3. Subcellular localization of human PRDX5 in yeast. A: WT-p*PRDX5*, WT-p*MITO*-PRDX5, WT-Vector and *ahp1*Δ-Vector strains were cultivated in SD-URA medium before immunofluorescence detection of PRDX5. Panels a–d: localization of human PRDX5; panels e–h: DAPI staining of nuclei of yeast illustrated in panels a–d. B: WT-p*MITO-COX4-PRDX5* strain was cultivated in SD-URA medium before immunofluorescence detection of PRDX5. Panel a: localization of human PRDX5 in yeast cells; panel b: Red MitoTracker staining of mitochondria of yeast cells illustrated in panel a; panel c: DAPI staining of nuclei of yeast cells illustrated in panels a and b. Bars: 10 μM.

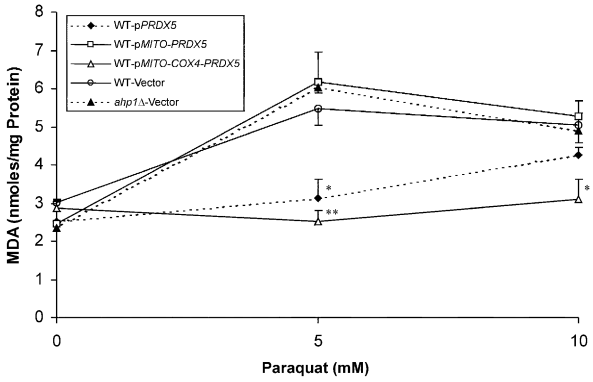

Fig. 4. Levels of MDA measured by the TBARS method in WT-p*PRDX5*, WT-p*MITO*-PRDX5, WT-p*MITO-COX4-PRDX5*, WT-Vector and *ahp1*Δ-Vector strains after a 3 h incubation with 0, 5 or 10 mM paraquat in SD-URA medium. The results are the means ± S.E.M. of three independent measurements. $*P < 0.05$ compared with WT-Vector; $**P < 0.01$ compared with WT-Vector.

3.3. Human PRDX5 protects yeast cells from paraquat toxicity

Increasing concentrations of paraquat in YPD solid medium produced different effects on the five yeast strains. At concentrations of 8 and 10 mM paraquat, WT-p*MITO-PRDX5*, WT-Vector and *ahp1*Δ-Vector showed almost no growth (Fig. 5, lanes 2, 4 and 5) whereas WT-p*PRDX5* and WT-p*MITO-COX4-PRDX5* grew (Fig. 5, lanes 1 and 3). The latter strain was the most resistant to paraquat.

4. Discussion

Recently, more attention was focused on PRDX roles in mammals as regulators of redox-sensitive signaling due to their moderate catalytic efficiency compared with those of classical glutathione peroxidases and catalase [9]. However, the specific subcellular localization of certain PRDXs in organelles that are sources of ROS, their abundance in many different cell types in mammals and recent findings that specific inhibition of individual PRDXs renders cells more sensitive to H_2O_2 toxicity, lipid peroxidation and apoptosis argue that mammalian PRDXs are also important protective antioxidant enzymes [14,29,30]. Therefore, to gain insight into protective antioxidant properties of the recently discovered human PRDX5, we expressed this thioredoxin peroxidase in mitochondria and in the cytosol of *S. cerevisiae* exposed to

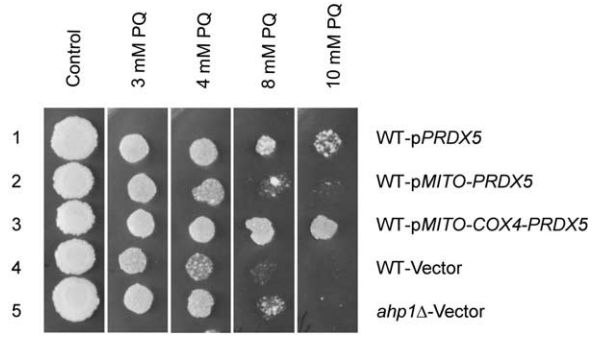

Fig. 5. Sensitivity of yeast strains to paraquat (PQ). WT-p*PRDX5*, WT-p*MITO*-PRDX5, WT-p*MITO-COX4-PRDX5*, WT-Vector and *ahp1*Δ-Vector strains were spotted on YPD plates containing different PQ concentrations. Growth was monitored after 3 days at 30°C.

oxidative stress induced by the herbicide paraquat, a redox active drug known to generate $O_2^{\bullet-}$ which can lead to the formation of H_2O_2 and hydroxyl radical ($^{\bullet}OH$) [18].

In mammals, PRDX5 can be addressed to mitochondria, peroxisomes and the cytosol [11,12,14]. Here, we show that human mitochondrial PRDX5 with its human mitochondrial presequence (MITO-PRDX5) and the short cytosolic/peroxisomal PRDX5 with its human peroxisomal targeting sequence (PRDX5) did not enter either into mitochondria or into peroxisomes in yeast. On the contrary, both proteins remain in the cytosol. These results demonstrate that mitochondrial and peroxisomal targeting sequences of human PRDX5 are not functional in *S. cerevisiae*. Furthermore, the lack of cleavage of the human mitochondrial presequence prevented the protective activity of PRDX5 against paraquat toxicity possibly by altering protein conformation. There are several reports in the literature showing that human mitochondrial presequences may not be fully functional in yeast [31]. Also, the mammalian C-terminal peroxisomal targeting sequence Ser-Gln-Leu (SQL), shared by human PRDX5 and rat pristanoyl–CoA oxidase, was demonstrated previously to be non-functional in yeast [32]. Therefore, to address human PRDX5 into yeast mitochondria, we performed a fusion between short PRDX5 and the *S. cerevisiae* COX4 mitochondrial presequence (MITO-COX4-PRDX5). The fusion protein was effectively targeted into yeast mitochondria and was correctly cleaved upon internalization into the organelle even if a proportion of uncleaved protein was still detected in immunoblots.

In this report, we show that the expression of short cytosolic PRDX5 and mitochondrial MITO-COX4-PRDX5 conferred higher resistance of yeast cells to paraquat. The toxic effect of paraquat is believed to be mediated by $O_2^{\bullet-}$ which is produced following enzymatic reduction of paraquat at the expense of NADPH or NADH. These reactions occur in mitochondria under the catalytic activity of NADH–cytochrome b5 oxidoreductase and NADH–ubiquinone oxidoreductase, and in the endoplasmic reticulum catalyzed by NADPH–cytochrome P450 oxidoreductase [19]. $O_2^{\bullet-}$ generated by the reduction of paraquat is then dismutated by mitochondrial MnSOD and cytosolic Cu/ZnSOD into H_2O_2. Unlike $O_2^{\bullet-}$, H_2O_2 is able to diffuse through the cytosol and can generate the highly toxic $^{\bullet}OH$ by Fenton and Haber–Weiss reactions [33]. Our results suggest that human cytosolic PRDX5 expressed in yeast cytosol is able to detoxify H_2O_2 coming from the cytosol and probably also from mitochondria. Moreover, human PRDX5 expressed in mitochondria was able to protect yeast cells even more efficiently than cytosolic PRDX5 from an oxidative stress caused by paraquat as shown by yeast cultures on plates but also as measured by the level of lipid peroxidation in liquid cultures. However, although these results suggest that a better protection is conferred by human PRDX5 localized in mitochondria, further studies would be necessary to determine whether this is related to enzymatic activity as PRDX5 levels were higher in mitochondria than in the cytosol as demonstrated by immunoblotting. It must be pointed out that human PRDX5 levels expressed in yeast were, in our experiments, in the physiological range of mammalian PRDXs (ng/μg total soluble protein) [14].

Interestingly, in this study, we have also shown that paraquat is equally cytotoxic for the *ahp1*Δ mutant and WT yeast as reported by Park et al. [17]. This lack of paraquat sensitivity for a yeast strain disrupted for the *S. cerevisiae* ortholog

of human PRDX5 could reflect a different catalytic efficiency of Ahp1p compared to human PRDX5. Indeed, recent data show that PRDX5 reductase activity towards H_2O_2 is higher than that of Ahp1p [14,17]. Moreover, an in vitro comparison of reductase activities of Ahp1p and cTPxI, another yeast PRDX, showed that whereas Ahp1p activity is specific for organic peroxides, cTPxI activity is specific for H_2O_2 [34]. Thereby, H_2O_2 formed in WT or *ahp1*Δ yeast by paraquat would be mainly reduced by cTPxI, which is a key antioxidant enzyme to protect yeast cells against intracellular oxidative stress, especially when induced by H_2O_2 [17].

In conclusion, we used the yeast *S. cerevisiae* as a cellular model to investigate the protective role of human PRDX5 against oxidative stress induced by paraquat. We provide, for the first time, evidence that mitochondrial and cytosolic human PRDX5 are able to protect eukaryotic cells against highly toxic paraquat.

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