Distinct Physiological Functions of Thiol Peroxidase Isoenzymes in Saccharomyces cerevisiae

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A new type of peroxidase (“thiol peroxidase”, TPx) having cysteine as the primary site of catalysis has been discovered from prokaryotes to eukaryotes. In addition to two yeast TPx isoforms (TSA I and TSA II/AHPC1) previously described, three additional TPx homologues were identified by analysis of the open reading frame data base for Saccharomyces cerevisiae. Three novel isoforms showed a distinct thiol peroxidase activity supported by thioredoxin, and appeared to be distinguishably localized in cytoplasm, mitochondria, and nucleus. Each isoform was named after its subcellular localization such as cytoplasmic TPx I (cTPx I or TSA I), cTPx II, cTPx III (TSA II/AHPC1), mitochondrial TPx (mTPx), and nuclear TPx (nTPx). Their transcriptional activities suggest that cTPx I and cTPx III are the most predominant isoforms among the five type isoforms. Transcriptional activities of TPx isoenzymes during yeast life span were quite different from each other. Unlike other TPx null mutants, cTPx I null mutant was hyper-sensitive to various oxidants except for 4-nitroquinoline N-oxide. The null mutant was more resistant toward 4-nitroquinoline N-oxide and acidic culture than its wild type. The severe growth retardation of cTPx II mutant resulted in accumulation of G1-phased cells. Based on kinetic properties of five isoforms, their subcellular localizations, and distinct physiology of each null mutant, we discussed the physiological functions of five types of TPx isoenzymes in yeast throughout the full growth cycle.

The incomplete reduction of molecular oxygen during respiration, lipid metabolism in peroxisomes, and aerobic metabolism cause to form reactive oxygen species (ROS)1 such as H2O2, alkyl hydroperoxides, and superoxide anion. ROS are potent oxidant capable of damaging all cellular components including DNA, protein, and membrane lipid. To protect against the toxicity of ROS, aerobic organisms are equipped with an array of defense mechanisms (1). Among these, a new type of peroxidase, named thiol peroxidase (TPx) has been known to eliminate H2O2 and alkyl hydroperoxides with use of a thiol-reducing equivalent (2, 3). The TPx family, also referred to as TSA/AhpC family, is a large family of newly coming peroxidases that have been discovered from prokaryotes to eukaryotes (4–9). Instead of active site of selenocysteine within glutathione peroxidase, they have one conserved cysteine within TPx as a primary site of catalysis. In mammalian tissue, at least, five types of TPx isoenzyme were identified (5). However, based on cellular compartmentalization and tissue distribution, their cellular functions remain still debatable. Recently, two types of TPx isoenzymes were reported to exist in Saccharomyces cerevisiae referred as TSA I (4, 10, 11) and TSA II/AHPC1 (12, 13). TSA I and TSA II/AHPC1 were described as a general hydroperoxide peroxidase to remove H2O2 and alkyl hydroperoxide (12, 13).

To gain insight into the physiological role of each TPx isoenzyme, we explored the function of the five isoforms in S. cerevisiae as a model organism for eukaryotes. In this paper, in addition to TSA I and TSA II/AHPC1, we reported for the first time the distinct subcellular localization and physiological function of three new types of TPx isoenzymes in yeast cells. Based on the different subcellular localization of TPx isoenzymes, their characteristic kinetic properties, and the physiological mutants lacking each TPx isoenzyme, we suggest a distinct physiological role of each TPx isoenzyme in maintaining aerobic life of yeast.

EXPERIMENTAL PROCEDURES

Yeast Culture and Media—Standard methods were used for growth, transformation, and genetic manipulation of S. cerevisiae (14). The S. cerevisiae strain JD7–7C (Mata, ura3–52, leu2, trpA, K+ ) was the recipient in the TPx gene disruption and GFP expression experiment.

Recombinant DNA Techniques and Other Methods—Standard protocols were used for all recombinant DNA techniques. Northern blot analysis was performed as described (15). RNA was isolated from log-phase cells by using a hot phenol extraction method (16). Total RNA (25 μg) was fractionated in a 1% denaturing agarose gel, blotted, and probed with PCR-amplified labeled ORF by using standard method (15). For substitution of functional cysteine residue of TPx, PCR-based strategy was employed to introduce nucleotide substitution at the defined location (17). For replacement of putative functional cysteine of TPx with serine, respective cysteine codon (TGT for cTPx II and nTPx, TGC for mTPx) was changed to AGT or AGC (serine). Patch assays were done as described below. Aliquots (10 μl) containing approximately 106 or the indicated cell number of an overnight culture were spotted on YPD plates containing oxidants at the indicated concentration. Plates were monitored after 2–5 days of incubation at 30 °C. β-Galactosidase assay was performed as described (18).

Plasmid Construction for Expression of Five Yeast TPx—Five yeast TPx genes were amplified from yeast genomic DNA by the polymerase chain reaction (PCR) with two pairs of primers covering whole coding sequence. Forward primer has an NdeI site and reverse primer has a BamHI site for cloning. The following primers were used for the PCR of TPx genes and enzyme sites are underlined: cTPx I (5’-CGATCCATAT-GGTGCCTCAAGTTCAAAAG (forward) and 5’-CGCGGATCCTTATTT-GTTGGCAGCTTCCGA (reverse)), cTPx II (5’-CGATCCATAT-GGTGCCTCAAGTTCAAAAG (forward) and 5’-CGCGGATCCTTATTT-GTTGGCAGCTTCCGA (reverse)).

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Expression of Recombinant TPx Proteins—Transformed cells were cultured at 37 °C overnight in LB medium supplemented with ampicillin (100 μg/ml) and transferred to fresh medium at the ratio of 1 to 200. When the optical density of the culture at 600 nm reached 0.4, isopropyl-1-thio-β-D-galactopyranoside was added to final concentration of 0.5 mM. After incubation for 3 h, cells were harvested by centrifugation and stored at −70 °C until use.

Frozen cells were suspended in 50 mM Tris-Cl (pH 8.0) containing 2 mM phenylmethylsulfonyl fluoride and 1 mM EDTA and disrupted by sonication. The supernatants after centrifugation at 18,000 rpm using SS-34 rotor were loaded to Q-Sepharose column that had been previously equilibrated with 50 mM sodium acetate (pH 5.0). The fractions corresponding to the peak of TPx protein were collected.

Construction of TPx-Green Fluorescent Protein (GFP) Chimeras—TPx was expressed with the same forward primer for pYGFP construction and a reverse primer (5'-AGATCTTTTGTATAGTTCATCCATGC) having BamHI and KpnI sites. The PCR product was ligated with expression vector pT7-7.

Determination of Thiredoxin-linked Peroxidase Activity of TPx—The reaction mixture containing 0.1 M Hepes-NaOH (pH 8.0), 1.0 μg of GS, 5 μM FeCl₃, various concentrations of TPx, and 10 mM pH DTT was incubated at 37 °C, and then 0.5 μl of γ-glutamyltransferase assay mixture was added. After incubation at 37 °C for 10 min, the remaining activity of GS was determined by measuring the absorbance at 540 nm.

Determination of Antioxidant Activity of TPx—Antioxidant activity was determined by measuring the activity to protect the inactivation of E. coli glutamine synthetase (GS) by a thiol metal-catalyzed oxidation (DTT/Fe²⁺/O₂) (thiol MCO system) as described previously (4).

Expression of Five Thiol Peroxidases in Yeast—Expression vectors were constructed with other forward primers (5'-AGAGTCTTTTGTATAGTTCATCCATGC) and reverse primers (5'-GAATTCATATGTCTGA-CAATTTTTGAAATAC) having BamHI and HindIII sites. For insertion of promoter at appropriate sites within pYGFP, the final PCR product was ligated with expression vector pT7-7.
TPx homologues (i.e., YDR453C, YBL064C, and YIL010W) were identified by inspection of the data base of *S. cerevisiae*. It was previously reported that the transcriptional level of YDR453C was induced by skn7, Yap1, and *H₂O₂* (22, 23). However, their biochemical and cellular functions were not investigated. Fig. 1 shows the primary structures of TPx homologous proteins. The amino acid sequence similarity among yeast TPx homologous proteins was 11.5%. The alignment of amino acid sequences showed one highly conserved cysteine within the TPx homologues, which suggested that three TSA homologues (i.e., YDR453C, YBL064C, and YIL010W) would be new members of TSA/AhpC family. The conserved cysteine within TSA I (YML028W) (i.e., Cys-48) and TSA II/AHP1 (i.e., Cys-62) was previously reported to act as primary catalysis (11–13). To characterize three putative TPx genes as thiol peroxidase, we cloned the genes and overexpressed the genes in *E. coli*. The proteins were homogeneously purified from the soluble fraction of the *E. coli* recombinants. The purities of proteins were confirmed on SDS-PAGE gels (Fig. 2). The purified proteins were heated at 95 °C for 5 min in SDS sample buffer with DTT or without DTT, and analyzed by SDS-PAGE. The analysis of gene products on the SDS-PAGE gel indicated that except for YIL010W all proteins existed as homodimers in the absence of DTT, suggesting that the monomers were linked by disulfide bond. However, YIL010W was detected at the molecular size corresponding to the monomeric form regardless of the presence or absence of DTT. This result suggests that like a mammalian TSA/AhpC family, ORF6 (28), YIL010W exist as monomer upon oxidation.

**Cellular Compartmentalization of TPx Isozyme—**Analysis of the primary structures of TPx isozymes using a program PSORT (29) for prediction of putative targeting signal sequences reveals that a 29-kDa TPx, YBL064C, harbors a mitochondrial localization signal in its N-terminal region (SFHRC-SAQLKRT) (30). YIL010W has a nuclear localization signal in its N-terminal region (KKKKK) (30). YL109W (i.e., TSA II/AHP1) (12, 13) has a peroxisomal signal sequence in its C-terminal region (172AHL) (170). However, no targeting signal was identified in both YML028W (TSA I) and YDR453C. The positions of the putative signals for targeting were shown in Fig. 1. In order to evaluate the putative targeting signals, we expressed the GFP fusion proteins of the five types in *S. cerevisiae*. The cellular compartmentalization of each fusion protein was determined by confocal fluorescence microscopy of intact yeast cells. The control GFP was evenly distributed in the cytoplasm as previously reported (Fig. 3A) (30). Each C-terminal YBL064C and YIL010W were fused to the N-terminal of GFP, whereas N-terminal YML028W, YDR453C, and YL109W were fused to the C-terminal of GFP. GFP-YML028W and GFP-YDR453C fusion proteins appeared to exist in the cytoplasm (Fig. 3, B and C). As expected, YBL064C-GFP and YIL010W-GFP appeared...
to be localized in the mitochondria (Fig. 3E) and the nucleus (Fig. 3G), respectively. To confirm this subcellular localization, we deleted the respective signal sequence in each protein, and fused to GFP. The resulting YBL064C'-GFP and YIL010W'-GFP recombinant proteins were evenly distributed in the cytoplasmic space (Fig. 3, F and H). Fig. 4, A and B, shows the mitochondrion and nucleus of yeast visualized by the organelle-specific fluorescent dyes. The exact overlapping between organelle-specific fluorescence and the corresponding GFP fusion protein fluorescence confirmed the mitochondrial and nuclear localization of YBL064C and YIL010W, respectively. However, in the case of GFP-YLR109W, the recombinant protein was evenly distributed in the cytoplasm despite of the presence of a putative peroxisomal signal sequence in its C-terminal region (174AHL176). (Fig. 3D). The localization of YLR109W in cytoplasm was also investigated by direct immunoblot analysis of the organelle-free soluble and peroxisomal fractions, which was obtained from the yeast maximally grown in YPD medium. The immunoblot analysis exerted that the cytosol fraction after ultracentrifugation at 100,000 g possessed TSA II/Ahp1 (YLR109W), whereas the precipitate containing peroxisome did not, suggesting its cytoplasmic localization. In contrast to our result, the possible peroxisomal localization of TSA II/Ahp1 was previously suggested (13).

Now, we renamed TPx isoenzymes, TSA I (YML028W), YDR453C, TSA II/Ahp1 (YLR109W), YBL064C, and YIL010W as cytoplasmic TPx I (cTPx I), cytoplasmic TPx II (cTPx II), cytoplasmic TPx III (cTPx III), mitochondrial TPx (mTPx), and nuclear TPx (nTPx) according to their subcellular localizations, respectively. The cytosolic localization of TSA I was also previously described (11).

Enzymatic Characterization of TPx Isoenzymes from S. cerevisiae—We carried out the enzymatic characterization of three novel TSA/AhpC proteins (cTPx II, mTPx, and nTPx). In the case of mTPx, the structural gene without DNA fragment encoding the signal sequences was expressed to obtain native proteins. A thiol-specific modification reagent, N-ethylmaleimide completely inactivated the antioxidant activities of the three novel proteins only in the presence of DTT, but did not without the preincubation of DTT (data not shown). This result suggests that three novel proteins may be thiol peroxidase. To investigate this possibility, we replaced each putative functional cysteine (Cys-48 for cTPx II, Cys-91 for mTPx, and Cys-107 for nTPx) with serine. All mutant proteins resulted in complete loss of antioxidant activity (data not shown). This result suggests that cTPx II (YDR453C), mTPx (YBL064C), and nTPx (YIL010W) are the novel members of AhpC/TSA family, and, at least, five types of TPx isoenzymes exist in S. cerevisiae.

We carried out a comparative study on the antioxidant activities exerted by the five types of TPx isoenzymes with DTT (Fig. 5A) and GSH (Fig. 5B) as a provider of reducing equivalents. The DTT-supported antioxidant activity of GS protection on pH 7.0 significantly varied with the types of TPx in the order of mTPx > cTPx I > cTPx II > cTPx III > nTPx. The GSH-dependent antioxidant activity given by each protein did not follow the order of the DTT-supported antioxidant activity. The GSH-dependent antioxidant activity of cTPx II was much lower than those of the other isoenzymes. This result suggests that all TPx proteins except for cTPx II can utilize GSH as the electron donor. In contrast to other TPx proteins, the very low GSH-linked antioxidant activity of cTPx II could be taken as evidence supporting the different environment of the active site of cTPx II from those of other TPx proteins.

We also examined the pH-dependent antioxidant activity of each TPx isoenzyme. The activities of cTPx I and cTPx II were not significantly affected by pH variation from 6.5 to 7.5, but the activities of the other TPx proteins significantly increased by changing the reaction pH from 7.5 to 6.5 (Fig. 5A). Especially, the antioxidant activity of mTPx was so highly sensitive to the change of the reaction pH that the activity was completely abolished at alkaline pH. This result suggests that the activity of mTPx could be regulated by cellular pH.

Peroxidase activities of TPx isoenzymes were comparatively investigated. Peroxidase activity was indirectly measured in terms of the increase of NADPH consumption by the thioredoxin system in the presence of peroxides. All TPx proteins
exerted significant thioredoxin-linked Px activity even though the extent of peroxidase activity remarkably varied with the type of protein (data not shown). The specific peroxidase activities of five types of TPx isoenzymes toward various peroxides were summarized in Fig. 4C. Among the five types of TPx proteins, cTPx I possessed the most potent capability to remove \( \text{H}_2\text{O}_2 \), whereas cTPx III possessed the most potent peroxidase activity toward organic hydroperoxide such as \( \text{t}-\text{butyl hydroperoxide} \) and cumene hydroperoxide. In addition, the peroxidase activity of cTPx III toward alkyl hydroperoxides was similar to that toward \( \text{H}_2\text{O}_2 \), whereas the peroxidase activity of cTPx I toward \( \text{H}_2\text{O}_2 \) was 4–5-fold higher than that toward organic hydroperoxides. The peroxide-selective peroxidase activities of cTPx I (TSA I) and cTPx III (TSA II/AHP1) were consistent with previous results (12). The peroxide selectivity of cTPx II, mTPx, and nTPx revealed that like cTPx I, cTPx II and mTPx preferentially reduced \( \text{H}_2\text{O}_2 \) rather than alkyl hydroperoxides, whereas nTPx showed the reverse selectivity as cTPx III. The extent of peroxidase activity, taken together with peroxide selectivity of each TPx, suggests that cTPx I and cTPx III act as a general hydroperoxide peroxidase to remove both \( \text{H}_2\text{O}_2 \) and alkyl hydroperoxide.

The thioredoxin-dependent Px activity of mTPx at pH 7.0 was much lower than that of cTPx I and cTPx III. However, the antioxidant activity of mTPx to protect the oxidative damage of glutamine synthetase caused by a metal-catalyzed oxidation (MCO) system (4) was much higher than that of cTPx I and cTPx III. This electron donor specific activity of mTPx suggests that thioredoxin could act as the poor electron. If it did not, peroxide was not the true substrate for mTPx. To investigate this issue, we directly measured the peroxidase activity of mTPx in terms of decrease of \( \text{H}_2\text{O}_2 \). Fig. 5D shows the Px activity of mTPx as a function of \( \text{H}_2\text{O}_2 \) concentration. The peroxidase activity profile as a function of \( \text{H}_2\text{O}_2 \) concentration revealed that mTPx appeared to be much more profoundly inactivated by increasing \( \text{H}_2\text{O}_2 \) concentration than cTPx I and cTPx III. The inactivation of cTPx I (TSA I) by \( \text{H}_2\text{O}_2 \) was described previously (31). Therefore, the contradictory activity of mTPx could be explained in terms of more severe inactivation of mTPx by \( \text{H}_2\text{O}_2 \) because of usage of excess concentration of \( \text{H}_2\text{O}_2 \) (1 mM) in the assay system for thioredoxin-linked Px activity. The physiological meaning of the various inactivation of each TPx isoenzyme by \( \text{H}_2\text{O}_2 \) remains unsolved. Taken together, these results suggest that all types of TPx can act as thi peroxidase supported by thioredoxin as a physiological electron donor.

Changes of Transcription of TPx—To investigate the inducibility of TPx in response to various oxidative stress, cells transformed with \( \text{TPx-LacZ} \) fused plasmid were exposed to various oxidants. The level of transcription of TPx was measured in terms of changes of the expression level of \( \beta\text{-galactosidase} \) gene (\( \text{lacZ} \)) whose promoter region was replaced by each TPx regulatory region (Fig. 6B). In aerobically cultured cells, the level of \( \beta\text{-galactosidase} \) activity was consistent with cellular level of mRNA for each TPx determined by Northern blot (Fig. 6A). These results, collectively, could be taken as the evidence supporting that cTPx I and cTPx III are predominant proteins among the five isoenzymes.

\( \text{TPx} \) transcriptional activities increased 2–5-fold by shifting anaerobic to aerobic cultures (Fig. 6B). Also, the levels increased significantly after the treatment of aerobically growing yeast with oxidative stress-inducing agents such as \( \text{H}_2\text{O}_2 \) and diamide. The level of cTPx II transcription was much lower than those of cTPx I and cTPx III in aerobic culture, but increased remarkably up to 7-fold after treatment with the oxidants, suggesting the highest inducibility of cTPx II. Be-
cause the transcriptional level of nTPx was extremely low compared with those of the other TPxs, the inducibility of nTPx could not be determined. This result suggests that at least four types of TPxs (cTPx I, -II, -III, and mTPx) are inducible proteins in response to oxidative stress.

The full growth cycle of yeast begins with log phase and progresses through the diauxic shift (i.e., the metabolic shift from fermentation to respiration) to true stationary phase (13). The metabolic and genetic control of gene expression on a genomic scale, in which the expression level of the mTPx (YBL064C) gene was activated after the diauxic shift as described (24). Therefore, the elevation of transcription of mTPx at diauxic shift provides rationale for its localization and function as a mitochondrial antioxidant. The level of transcription of cTPx I remained constant before the diauxic shift, after which it increased slightly, whereas those of cTPx II and cTPx III gradually increased as yeast cells grew. The gradual increase of all TPx transcriptions as a function of culture time suggests the inducibility of TPx genes in response to oxidative stress imposed during growing. The different transcriptional activity of TPx promoters during yeast life span was taken as the evidence for different subcellular location and physiology of each TPx isoenzyme.

Physiology of TPx Null Mutants—The growth responses of the mutants deficient in each TPx isoenzyme (cTPxΔI, cTPxΔIII, mTPxΔ, and nTPxΔ) toward various oxidants were examined. Among the null mutants, only cTPxΔI showed a broad spectrum of hypersensitivity toward oxidative stresses caused by H₂O₂, t-butyl hydroperoxide, and diamide (Fig. 7). The growth of cTPxΔIII was specifically retarded by t-butyl hydroperoxide (Fig. 7). The alkyl hydroperoxide-specific effect on cTPxΔIII was consistent with the result previously reported (13). The organic hydroperoxide-specific sensitivity of cTPxΔIII could be explained in terms of its alkyl hydroperoxide-selective kinetic properties. At high concentrations of diamide (0.5 mM), all mutants grew less than wild type, suggesting their antioxidant roles in vivo. Taken together, these results suggest that cTPx I acts as critical antioxidants in vivo. The potential antioxidant role of cTPx I could be easily expected from its predominant existence in the cytoplasm and relatively strong antioxidant activity.

In contrast to the other TPx mutants and their parent type, cTPxΔI and cTPxΔIII showed an unusual physiology. The mutant lacking cTPx I exerted an abnormal physiology such as resistance to 4-nitroquinoline N-oxide and acidic pH (Fig. 8). To see that the abnormal physiology of cTPxΔI resulted from oxidative stress caused by deleting cTPx I, the extent of oxidative stress imposed on the mutant was examined. As an indicator for cellular oxidative stress, the level of cTPx III in cTPxΔI was compared with that of cTPx I in the mutant lacking cTPx III. The analysis on Western blot (data not shown) and two-dimensional gels shown in Fig. 9 revealed that cTPx I and cTPx III were significantly induced by H₂O₂. And also the level of cTPx III in the cTPxΔI was increased to the same extent as that of wild type induced by H₂O₂. However, the level of cTPx I in cTPxΔIII was not significantly increased with comparison to that of its wild type. This result, taken together with its potent peroxidase activity, suggests that cTPx I may be an important antioxidant to regulate cellular oxidative stress. Thus, this abnormal physiology of cTPxΔI can be taken as evidence for the accumulation of oxidative stress caused by deletion of cTPx I.

At first glance, Fig. 8 showed that cTPxΔII mutant appeared to be hypersensitive toward oxidative stress. However, the same viability of cTPxΔII as that of wild type against diamide (Fig. 8B), taken together with the severe growth retardation on control plate (Fig. 8A), exerted that the growth retardation of cTPxΔII did not result from oxidative stress given by oxidants. To investigate this issue, the growth rates of TPx mutants and wild type yeast were determined under aerobic conditions. Fig.
A shows the growth rates of TPx null mutants. The order of growth rate for each mutant and wild type as follows: wild type, cTPx III, nTPx, mTPx, cTPx II. To confirm the growth retardation of cTPx II, all mutants grew to higher mass for 48 h (Fig. 10B). As expected, cTPx II did not recover the growth during the longer cultivation, suggesting the possible involvement of cTPx II in yeast growth. To rule out the possibility that the slow growth of cTPx II simply resulted from the highly imposed oxidative stress by deletion of cTPx II, which, in turn, could affect the cell growth via lowering viability, the sensitivity of TPx II toward oxidative stress was tested. We previously reported that the growth retardation of cTPx I in aerobic culture recovered in anaerobic culture (11), which was taken as evidence for the antioxidant role of cTPx I. However, in contrast to the case of cTPx I, the severe growth retardation of cTPx II in aerobic culture did not recover in the anaerobic culture (Fig. 10C), suggesting that the growth retardation of cTPx II may not result from the oxidative stress caused by deleting cTPx II. This result was consistent with the same viability of cTPx II as that of wild type revealed by the spot test (Fig. 8B). In contrast to those of other mutants, the number of cTPx II colony on plate containing diamide was
Samples were taken and null mutants. as asynchronous cultures of wild type imposed elevated as much oxidative stress as that of wild type. Deletion of cTPx I mutant was significantly retarded. Deletion of cTPx II exerted the hypersensitivity toward various oxidants. Among the five null mutants, "housekeeping" type of antioxidant protein capable of removing such as H2O2 and paraquat, and diamide. The aerobic growth of cTPx I mutant was equivalent to that of wild type (Fig. 11). This result suggests that the slow-growth phenotype of cTPx II on yeast cells greatly increased the cellular oxidative stress. The distinct physiology of cTPx I mutant suggests that cTPx I is a key antioxidant to regulate intracellular oxidative stress, especially H2O2.

**Severe Growth Retardation of TPx II Null Mutant**—Although the inducibility of cTPx II by oxidative stress was about 5-fold greater than that of cTPx I, the induced level of cTPx II was found to be still much lower than that of cTPx I, and the null mutant showed a much lower sensitivity toward oxidants than cTPx I. Deletion of cTPx II resulted in dramatic retardation of yeast growth and a profound increase in G1 population compared with that of wild type, while the cell cycle pattern of cTPx II, which was hypersensitive toward various oxidant, was equivalent to that of wild type. These somewhat contradictory results might be taken as evidence supporting a physiological role for cTPx II in cell proliferation. This speculation could be supported by a previous report (39) that the human PAG gene product, one member of TSA family, which is an inhibitor of the c-Abl tyrosine kinase, is overexpressed in cells entering S phase and by contact with agents inducing oxidative stress. PAG associates with c-Abl in vivo and inhibits tyrosine phosphorylation induced by overexpression of c-Abl (40).

**Cytoplasmic TPx III Acts as an Important Cytoplasmic Alkyl

**Fluorescent Intensity**

**DISCUSSION**

**Cytoplasmic TPx I Acts as a Principal Antioxidant**—Based on the subcellular localization of five types of TPx isoenzymes in yeast cell, we discussed the physiological functions of TPx isoenzymes during the full life span. Predominant and constant transcriptional activity of TPx I in yeast cytoplasm during the full growth cycle suggests a physiological role of TPx I as a "housekeeping" type of antioxidant protein capable of removing various oxidative stresses. Among the five null mutants, cTPxII exerted the hypersensitivity toward various oxidants such as H2O2 and paraquat, and diamide. The aerobic growth of cTPx I mutant was significantly retarded. Deletion of cTPx I elevated as much oxidative stress as that of wild type imposed by H2O2. S. cerevisiae AP-1 (yAP-1) has known to be a key mediator of oxidative stress tolerance (32). Transcriptional activation by yAP-1 has been shown to be inducible by exposure of cells to H2O2 and diamide. The null mutant of yAP-1 has been shown to show hypersensitivity to H2O2 and diamide (33). It has been reported that in addition to hypersensitivity to H2O2 and diamide, the yAP-1 deletion mutant exerted abnormal resistance to 4-nitroquinoline N-oxide (34). Similarly, the cTPxII also exerted a higher tolerance against 4-nitroquinoline N-oxide than its wild type. Also, the cTPxII showed a higher acidic tolerance compared with its wild type. Recently, it has been reported that H2O2 protects yeast cells from inactivation by ionizing radiation (35), and activates plasma membrane H+-ATPase that is involved in the H+ extrusion from cytoplasm (36, 37). Brandao et al. (38) suggested a possible involvement of signaling pathway in activation of plasma membrane H+-ATPase in S. cerevisiae. Therefore, the abnormal physiologies of cTPxII can be taken as evidence supporting that deletion of cTPx I in yeast cells greatly increased the cellular oxidative stress. The distinct physiology of cTPx I mutant suggests that cTPx I is a key antioxidant to regulate intracellular oxidative stress, especially H2O2.

**Cytoplasmic TPx III Acts as an Important Cytoplasmic Alkyl

**Fig. 11.** Flow cytometry analysis of asynchronous cultures of wild type and null mutants. Samples were taken at A600 1–1.5 and at least 5000 cells from each sample were analyzed. First and second peaks represent G1-phased and G2-phased cell population, respectively. Panel 1 indicates wild type; panel 2, cTPxIII; panel 3, nTPxΔ; panel 4, cTPx ΔI; panel 5, nTPxΔ; panel 6, cTPx ΔIII. Two independent experiments were carried out with similar results.
Hydroperoxide Peroxidase—Subcellular localization of cTPx III indicates that it exists in the cytoplasm. In addition to predominant existence of cTPx III in the cytoplasm, the hypersensitivity of cTPxαIII toward alkyl hydroperoxides, not toward H$_2$O$_2$ suggests its important physiological function as an alkyl hydroperoxide peroxidase. In contrast to the other cytoplasmic isoenzymes (cTPx I and II), cTPx III is more active as an antioxidant at acidic pH. It has been known that as yeast is growing, the cytoplasmic pH is gradually shifted to acidic pH. During respiration, mitochondrial pH is shifted to acidic pH. The activity increases strikingly as the pH is decreased to acidic pH. The antioxidant activity of mTPx is very sensitive to pH. The activity increases strikingly as the pH is decreased to acidic pH. Therefore, the high inducibility and the resultant highest level of cTPx III among TPx isoenzymes in stationary-phase yeast suggest that cTPx III acts as an important cytoplasmic alkyl hydroperoxide peroxidase to maintain the yeast cells in stationary phase. Collectively, these results implicate the important physiology of cTPx III in stationary phase of yeast.

mTPx Is Localized in Mitochondria—In stationary phase yeast, most energy comes from mitochondrial respiration. The mitochondrial localization of mTPx indicates the physiological role of mTPx in yeast mitochondria. The production of ROS during respiration causes peroxidation of membrane lipids, cleavage of mitochondrial DNA, and impairment ATP generation, with resultant irreversible damage to mitochondria (41). It is worth noting that in contrast to cytoplasmic TPx (cTPx I and II), the antioxidant activity of mTPx is very sensitive to pH. The activity increases strikingly as the pH is decreased to acidic pH. During respiration, mitochondrial pH is shifted to acidic pH. Thus, at that acidic pH, the peroxidase activity of mTPx may be essential for scavenging ROS such as H$_2$O$_2$ evolved from mitochondrial respiration. The mitochondrial localization of mTPx could be supported by our finding that mTPx is highly synthesized at the late stationary phase in which the yeast energy metabolism shifts to respiration, which is consistent with the data from Metabolic Data Base (24). Therefore, the stationary phase in which the diauxic shift occurs, mTPx, should be physiologically important to thiol peroxidases to protect the oxidative damage of mitochondria caused by ROS produced from rapidly respiring mitochondria.

TPx Existing as well Componentalized Multiform Has a Distinct Physiological Role—Five isoenzymes of TPx are well compartmentalized in yeast cell. The peroxide selectivity of each TPx toward H$_2$O$_2$ and alkyl hydroperoxides is different from each other. Based on their peroxidase activities toward H$_2$O$_2$ and alkyl hydroperoxides, we can divide them into two groups such as hydrogen peroxide peroxidase (cTPx I, cTPx II, and mTPx) and alkyl hydroperoxide (cTPx III and nTPx). Cytoplasmic TPx I could act as a housekeeping type of peroxidase to regulate the intracellular ROS level such as H$_2$O$_2$. cTPx III could function as an alkyl hydroperoxide peroxidase especially in late stationary phase. The other new forms, mTPx and nTPx, are organelle-specific antioxidants acting as a peroxidase in mitochondria and nucleus, respectively. As far as we know, nTPx is the first reported thiol peroxidase to be located in the nucleus, which implies that nTPx could act as a peroxidase in nucleus. In nucleus, therefore, nTPx could protect oxidative damage of DNA by ROS. Based on intracellular localization, kinetic properties, the response of mutants to oxidative stress, and the physiology of yeast, we suggest that all of TPx isoenzymes are well designed to maintain the life of yeast cells throughout the full growth cycle.

ROS does not have exclusively toxic effects. Low levels of ROS can act as signaling molecule under physiological conditions (42). In mammals, ROS such as H$_2$O$_2$ produced in physiological conditions can activate transcription factor, such as NFκB and AP-1 (43), and can function as signals in apoptosis that are induced by tumor necrosis factor-α (44). There has been known that at least five types of TPx isoenzymes exist in mammalian cells. However, their intracellular localization and physiological functions remain poorly understood. Therefore, comparative studies between yeast and mammalian TPx isoenzymes will be helpful for understanding the physiological roles of TPx isoenzymes in mammalian cells. It remains that further studies aimed at understanding the abnormal growth retardation of cTPx II mutant are needed. Considering the in vivo function of TPx as a peroxidase, some TPx might act as a regulator for the cytoplasmic level of H$_2$O$_2$, which is suggested to act as second messenger as an in vivo oxidant to change the biological activities of transcription factors. Very similar sequence homology between cTPx II and human PAG (59.5% amino acid sequence identity) suggests that cTPx II may be a counterpart of mammalian proliferation-associated gene product (PAG), which is a member of the mammalian TSA family. Severe growth retardation of the cTPxαII mutant, taken together with the high transcriptional activity of cTPx II in response to oxidative stress, could explain why the PAG gene expression occurs in two cellular events: response to oxidative stress and proliferation (40).

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