GPX2, Encoding a Phospholipid Hydroperoxide Glutathione Peroxidase Homologue, Codes for an Atypical 2-Cys Peroxiredoxin in Saccharomyces cerevisiae*

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We have previously reported that Saccharomyces cerevisiae has three glutathione peroxidase homologues (GPX1, GPX2, and GPX3) (Inoue, Y., Matsuda, T., Sugiyama, K., Izawa, S., and Kimura, A. (1999) J. Biol. Chem. 274, 27002–27009). Of these, the GPX2 gene product (Gpx2) shows the greatest similarity to phospholipid hydroperoxide glutathione peroxidase. Here we show that GPX2 encodes an atypical 2-Cys peroxiredoxin which uses thioredoxin as an electron donor. Gpx2 was essentially in a reduced form even in mutants defective in glutathione reductase or glutaredoxin under oxidative stressed conditions. On the other hand, Gpx2 was partially oxidized in a mutant defective in cytosolic thioredoxin (trx1Δtrx2Δ) under non-stressed conditions and completely oxidized in tert-butyl hydroperoxide-treated cells of trx1Δtrx2Δ and thioredoxin reductase-deficient mutant cells. Alanine scanning of cysteine residues of Gpx2 revealed that an intramolecular disulfide bond was formed between Cys57 and Cys83 in vivo. Gpx2 was purified to determine whether it functions as a peroxidase that uses thioredoxin as an electron donor in vitro. Gpx2 reduced H2O2 and tert-butyl hydroperoxide in the presence of thioredoxin, thioredoxin reductase, and NADPH (for H2O2, Km = 20 μM, kcat = 9.57 × 103 s⁻¹; for tert-butyl hydroperoxide, Km = 62.5 μM, kcat = 3.68 × 103 s⁻¹); however, it showed remarkably less activity toward these peroxides in the presence of glutathione, glutathione reductase, and NADPH. The sensitivity of yeast cells to tert-butyl hydroperoxide was found to be exacerbated by the co-existence of Ca2+, that was most obvious in Gpx2 was not affected by Ca2+

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The abbreviations used are: GSH, glutathione; TRX, thioredoxin; GPX, glutathione peroxidase; cGPX, cytosolic GPX; PGPHX, phospholipid hydroperoxide GPX; gpGPX, gastro-intestinal GPX; pGPX, plasma GPX; eGPX, epididymis-specific secretory GPX; SeCys, selenocysteine; Prx, peroxiredoxin; TPx, thioredoxin peroxidase; r-BHP, tert-butyl hydroperoxide; AMS, 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid; WT, wild type; YPD, yeast extract/peptone/dextrose.

Compared with anaerobic organisms, aerobic organisms produce energy (ATP) more efficiently because of the electron transfer system in mitochondria. At the final step in the electron transfer process, molecular oxygen (O2) accepts four electrons and is reduced to H2O by cytochrome c oxidase. In the electron transfer system as well as normal metabolism in the cytoplasm, a portion of O2 remains in an insufficient state in reduction, which in turn is referred to as reactive oxygen species. Organisms of all types possess antioxidant systems, and the diversity of the systems is of interest in terms of understanding the evolution of organisms. Superoxide dismutase catalyzes the disproportionation of superoxide anion radicals (O2⁻) to O2 and H2O2, and has been found in a wide variety of organisms. Because H2O2 generated by the superoxide dismutase reaction is still reactive oxygen species, organisms have been armed with many antioxidant enzymes that can scavenge H2O2. Catalase decomposes H2O2 to H2O and O2, whereas peroxidases reduce H2O2 to H2O using various reducing power. For example, ascorbic acid is used by ascorbate peroxidase in plants, and glutathione (GSH) is used by glutathione peroxidase (GPx) in mammals. We have cloned three GPx homologue genes (GPX1, GPX2, and GPX3) from the budding yeast Saccharomyces cerevisiae (1). Phylogenetic analysis of each gene product revealed that yeast GPxs are similar to mammalian phospholipid hydroperoxide glutathione peroxidase (PHGPx) (2). In mammalian GPxs, selenocysteine (SeCys) is conserved at the active site of the enzyme, whereas in such GPx homologues Cys is conserved corresponding to the position of SeCys in mammalian GPxs (1). In the GPx reaction, oxidized SeCys (SeCys-OH) is reduced by glutathione (GSH), and the glutathione disulfide (oxidized glutathione, GSSG) thus formed is reduced to GSH by glutathione reductase using NADPH as a reducing power. It has been reported that the conversion of SeCys to Cys in mammalian GPxs reduces the enzyme activity drastically (3, 4); therefore, the catalytic mechanism of such non-SeCys-type GPx homologues, including yeast GPxs, is of considerable interest.

A series of peroxiredoxins referred to as peroxiredoxins (Prxs) have been found from lower prokaryotes to higher eukaryotes, and from Archaea, and they constitute a large family (5). There are several common features of Prxs, i.e. these enzymes do not have redox cofactors such as metal and prosthetic group and contain conserved Cys residue(s). Depending on the number of Cys residues conserved, Prxs are basically divided into two groups, 1-Cys Prx and 2-Cys Prx. In both types of Prx, the N-terminal Cys is absolutely conserved, which is sometimes called the "peroxidatic" cysteine (Cys-S•H) (6). In the 1-Cys Prx reaction, Cys-S•H is oxidized to cysteine sulfinic acid (Cys-SO2H) through the reduction of H2O2 to H2O, and the Cys-SO2H formed is reduced to Cys-S•H by an unknown reducing agent. In many 1-Cys Prxs, TRX serves as the endogenous reducing agent. By contrast to the 1-Cys Prx, the 2-Cys Prx has a second conserved Cys in the C terminus, which is sometimes called the "resolving" cysteine (Cys-S•H) (6). Because Cys-SO2H is unstable, it undergoes further oxidation to yield cysteine sulfenic acid (Cys-S•SO2H), the formation of which turns 2-Cys Prx inactive as a peroxidase. Hence, sulfiredoxin recovers Cys-S•O2H to Cys-S•H.
Alternatively, the Cys-S$_3$OH is attacked by the Cys-S$_3$H of another monomer to form intermolecular disulfide bonds, and these disulfide bridges are reduced by TRX. Due to the reducing agent in the peroxidase reaction, Prx is also referred to as the thioredoxin peroxidase (TPx). S. cerevisiae has five Prx/TPx genes, i.e. TSA1, TSA2, AHPI, DOTS, and PRX1 (7). The first four genes code for 2-Cys Prx, and the last codes for atypical 2-Cys Prx; however, again, the Gpx3 of homologue of GPx but not Prx.

Delaunay et al. (9) have recently reported that Gpx3 also functions as a redox transducer of Yap1, a transcription factor critical for oxidative stress response in S. cerevisiae (10). They proposed that Cys$^{36}$ of Gpx3, which corresponds to the position of Secys in mammalian Gpx (1), is oxidized to Cys$^{36}$-SOH by H$_2$O$_2$, and Cys$^{38}$ of Yap1, which is located in the C terminus cysteine-rich domain (c-CRD) overlapping the nuclear export signal of this b-ZIP transcription factor (11–13), attacks Cys$^{36}$-SOH to form a mix disulfide between both cysteines in Gpx3 and Yap1. Subsequently, the intermolecular disulfide bridge is reduced by Cys$^{38}$ of Yap1, which is located in the n-CRD, and consequently, the intramolecular disulfide bond within Yap1 is formed, and Gpx3 is reverted to the reduced form. Additionally, if Yap1 is absent, the Cys$^{36}$ within the same monomer of Gpx3 attacks Cys$^{36}$-SOH to form an intramolecular disulfide bond, and this disulfide bond is reduced by TRX. As a result, a TRX-dependent peroxidase reaction is accomplished. This mode of disulfide bridge formation during a peroxidase reaction (intramolecular disulfide bond) is the atypical 2-Cys Prx; however, again, the Gpx3 of S. cerevisiae is a homologue of Gpx but not Prx.

In the present study we demonstrate that Gpx2 is a atypical 2-Cys Prx. We show that the redox state of Gpx2 is maintained by a TRX-dependent system in vivo. We also show that the purified Gpx2 exhibits TRX-dependent peroxidase activity toward H$_2$O$_2$ and t-BHP in the presence of thiorredoxin reductase and NADPH in vitro but exhibits low peroxidase activity in the presence of GSH, glutathione reductase, and NADPH. The alanine scanning of cysteine residues of Gpx2 revealed that Cys$^{37}$ and Cys$^{38}$ seem to contribute to the formation of an intramolecular disulfide bond as a result of the peroxidase reaction. We also show that Gpx2 is important in the response to oxidative stress in the presence of calcium.

**EXPERIMENTAL PROCEDURES**

*Strains and Plasmids—*Basically, all S. cerevisiae strains used in this study have the genetic background of YPH250 (MATa trp1–1 his3–Δ200 leu2–Δ1 lys2–801 ade2–101 ura3–5). A trr1Δ mutant (MATa trp1–1 his3–11 leu2–3, 112 ade2–1 ura3–1 bar1 trr1Δ::HIS3) was described in Pearson and Merrill (14). The yeast strains used are summarized in **TABLE ONE**.

The construction of YEp13 + TRX1, YEp13 + TRX2, YEp13 + GRX1, and YEp13 + GRX2 was described previously (15). To clone the overexpression allele of Gpx2, the PCR was done with the primers (Gpx2–3 and GPR1–1) (1, 16) and the genomic DNA of wild-type cells. The PCR fragment was digested with SalI, and the resultant DNA fragment was cloned into the SalI site of pRS413 to yield pRSGPX2–3H.

**Cys Modification with 4′-Acetamido-4′-maleimidostilbene-2,2′-disulfonic acid (AMS)—**Cells were cultured in SD minimal medium (2% glucose, 0.67% yeast nitrogen base w/o amino acids supplemented with the appropriate amino acids and bases) at 28 °C. When the A$_{610}$ of the culture reached 1.0, peroxides (0.4 mM H$_2$O$_2$, 0.6 mM t-BHP, or 0.2 mM cumene hydroperoxide) were added. Cells were cultured for another hour at 28 °C, and five A$_{610}$ units of the culture for the wild type, gpx1Δgpx2Δ, grl1Δ, and trr2Δ cells or two A$_{610}$ units of the culture for trr1Δtrr2Δ, trr1Δtrr2Δtrr3Δ, and trr1Δ cells were collected by centrifugation. Cells were washed twice with 20% trichloroacetic acid solution and once with the acetone. After removal of acetone, cells were treated with AMS (Molecular Probes) as described previously (17).

Briefly, cells were suspended with 40 μl of sample buffer (80 mM Tris–HCl (pH 6.8), 2% SDS, 6 mM urea, 1 mM phenylmethylsulfonyl fluoride, and 0.05% bromphenol blue) containing 20 mM AMS. A small portion (~4 μl) of 1 M Tris–HCl (pH 8.0) was added to adjust the pH of the sample, and the mixture was boiled for 2 min. Twenty microliters of the sample was subjected to non-reducing SDS-PAGE followed by Western blotting using anti-Gpx2 antibody to determine the redox state of Gpx2.

**Western Blotting—**After SDS-PAGE, separated proteins were transferred to a polyvinylidene difluoride membrane (Immobilon, Millipore). Anti-Gpx2 antibody and anti-yeast TRX antibody raised in rabbits were used. After blocking with 5% nonfat dry milk, membranes were probed with anti-Gpx2 antibody to determine the redox state of Gpx2.

**Alanine Scanning of Cysteine Residues of Gpx2—**PCR-based site-directed mutagenesis was employed to introduce point mutations corresponding to the Cys residues of Gpx2. The first PCR was performed with the following primers: Gpx2–1 (5′-TTACCCTTGTCGACCTTGCTCTC-3′) (18) plus the mutagenesis primer (reverse primer; e.g. Gpx2–CA1R) and GPR1–1 (5′-GATCAAGCGTGTCGACATGCAAAC-AAGAGGC-3′) (1) plus the mutagenesis primer (forward primer; e.g. Gpx2–CA1-F). The primers used for mutagenesis were as follows: Gpx2–CA1F, 5′-GATTTAAGGCAAGGACAAAGAAG-3′; Gpx2–CA2F, 5′-TGAGGGAGCCGGGCCTTCAAGCC-3′; Gpx2–CA3F, 5′-AAGGACAAAGGACAAAGAAG-3′.
Redox Regulation of Yeast Gpx2 by Thioredoxin

TGGGTTCCGCGCCAATGTTCCG-3′; GPX2-CA4F, 5′-CGGATCCATGATATATGG-3′; GPX2-CA1R, 5′-CTTCT-TGTCTCCTGCTTCTAAATC-3′; GPX2-CA2R, GGCGTGAGGCC-GCCGTCGAGGCCA-3′; GPX2-CA3R, 5′-CGAATCGATGGGCGCGGAACCCCA-3′; GPX2-CA4R, 5′-CCATAATCTGCGC-AAATTCCG-3′; CA1, CA2, CA3, and CA4 correspond to Cys5, Cys7, Cys65, and Cys68, respectively. The last letter of each primer name represents the direction of the primer; i.e., F and R for forward and reverse, respectively. The plasmid carrying the promoter, open reading frame, and 3′-untranslated region of GPX2 was cloned into the plasmid pRS415 (pRS415–GPX2–1), of which the GPX2 gene was cloned by PCR with primers GPX2–1 and GPX2–1R, was used as template DNA. The PCR products amplified by each primer set were combined, subjected to a second PCR with GPX2–1 and GPX2–1R, digested with SacI (the corresponding sequence is underlined in GPX2–1 and GPX2–1R), and then introduced into the Sall site of pRS415 to generate a series of Cys → Ala mutants. The resultant plasmids were named pRS415–GPX2 C10A–1, pRS415–GPX2 C37A–1, pRS415–GPX2 C65A–1, and pRS415–GPX2 C83A–1. Each mutation was confirmed by DNA sequencing.

Expression and Purification of Gpx2 from Escherichia coli—The GPX2 gene containing a translational initiation codon and a stop codon was amplified with the following primers: GPX2-Nde, 5′-AAGTAATAATTCCATATGACCACATCTTT-3′; GPX2-Bam, 5′-TGGATATTATTATTGGATCGCAGTTGAATTATGG-3′; GPX2-CA4R, 5′-CCGAACTGATTGGC-CCATAATTCAACTGCGC-3′; GPX2-CA1R, 5′-CTTTCT-GCTTTGCTTCTAAATC-3′; CA1, CA2, CA3, and CA4 correspond to Cys5, Cys7, Cys65, and Cys68, respectively. The plasmid carrying the promoter, open reading frame, and corresponding description. Glutaredoxin is

RESULTS

Determination of Redox State of Gpx2 with AMS Modification Assay—If the peroxidase reaction is carried out by Gpx2, the sulfhydryl group of the active site Cys would be initially oxidized to cysteine sulfenic acid. To accomplish the catalytic cycle of the peroxidase reaction in vivo, there are two pathways to regenerate the sulfhydryl group at the active site Cys, i.e. (i) directly via an endogenous reducing agent such as GSH or TRX, or (ii) by generating a disulfide bond with another sulfhydryl group intramolecularly or intermolecularly and, subsequently, resolving the disulfide bridge thus formed with a protein-disulfide oxidoreductase such as TRX or glutaredoxin. We determined the redox state of Gpx2 in yeast cells using the AMS modification assay. AMS modifies the sulfhydryl group of a protein irreversibly. Because the molecular weight of AMS is ~500, the apparent molecular weight of Gpx2 will increase by 500 with every modification of a Cys residue in Gpx2. Indeed, because Gpx2 has four Cys residues, the complete modification of all Cys residues of Gpx2 with AMS (which means Gpx2 is in the reduced form) results in an increase of 2000 in the apparent molecular weight.

To verify the validity of the AMS modification assay, we measured the mobility shift of purified Gpx2 after treatment with AMS in non-reducing SDS-PAGE. The molecular weight of Gpx2 deduced from the amino acid sequence is 18,406. As shown in Fig. 1A, the molecular mass of unmodified Gpx2 was estimated to be ~18 kDa. After treatment with AMS, the apparent molecular mass was estimated to be 20 kDa, suggesting that all Cys residues of Gpx2 were modified with AMS. We confirmed this by titration of remaining Cys residues with 5,5′-dithio-bis(2-nitrobenzoic acid). Hence, the AMS-modified Gpx2, i.e. Gpx2 in reduced form (Gpx2red), migrated slowly in non-reducing SDS-PAGE.

Redox State of Gpx2 in Mutants Defective in Glutathione Recycling—In the Gpx reaction GSH is used as an endogenous electron donor, and oxidized glutathione (GSSG) is reduced by glutathione reductase. Therefore, if the recycling of GSH is impaired, the catalytic cycle of Gpx will be arrested. S. cerevisiae has a single glutathione reductase encoded by GLR1 (20), and therefore, the redox ratio of GSSG to total glutathione in gnr1Δ cells increased 3-fold compared with that of wild-type cells due to the impairment of the recycling of GSH (21). However, as shown in Fig. 1B, Gpx2 was essentially in the reduced form in wild-type as well as in gnr1Δ cells even under conditions of oxidative stress. The oxidized Gpx2 (Gpx2ox) in a trx1Δtrx2Δ mutant was shown as a control (details are described in Fig. 1C and corresponding description). Glutaredoxin is
FIGURE 1. Redox state of Gpx2 in vivo in different genetic backgrounds. A, AMS modification of the purified Gpx2. The purified Gpx2 was treated with AMS as described under “Experimental Procedures” and subjected to non-reducing SDS-PAGE followed by Western blotting. MW, molecular weight. B, effect of peroxides on the redox state of Gpx2. Cells were cultured in SD medium to log phase and exposed to peroxides as indicated in the figure for 1 h. Subsequently, Gpx2 was modified with AMS. Strains used in each lane are indicated in figure. C, effect of peroxides on the redox state of Gpx2 in mutants defective in TRX and its recycling system. Cells were cultured and exposed to peroxides as described in B. Strains used in each lane are indicated in the figure. Because the expression of Gpx2 is induced by oxidative stress in a Yap1-dependent manner (1, 18), the Gpx2 level is increased in cells treated with H2O2 or t-BHP. In addition, Yap1 is constitutively activated in trr1Δ mutants and trx1Δ mutants (15, 26). Basal levels of Gpx2 in these mutants are higher than those of other strains.

Redox State of Gpx2 in Mutants Defective in Thioredoxin—Besides glutaredoxin, TRX is also a small enzyme that functions as a protein-disulfide oxidoreductase. In many Prxs, TRX serves as the endogenous reducing power. In addition, some kinds of GPx have been reported to use TRX as a reducing power (22, 23). S. cerevisiae has three TRXs (TRX1, TRX2, and TRX3) (24, 25). Both Trx1 and Trx2 are basically cytosolic residents (15), whereas Trx3 is located in mitochondria (25). We disrupted each of the three TRXs and determined the redox states of Gpx2 in the resultant mutant, although no distinct difference in the redox state was observed in a single mutant of each TRX even under conditions of oxidative stress (data not shown, result for trx3Δ is shown in Fig. 1C). However, in a cytosolic TRX-deficient mutant (trx1Δtrx2Δ), a small proportion of Gpx2 was in the oxidized form under non-stressed conditions, and a large amount of Gpx2ox appeared after the treatment with H2O2 or t-BHP, and cumene hydroperoxide (Figs. 1B and 1C).

We examined the effect of H2O2 and t-BHP concentrations on the redox state of Gpx2 in trx1Δtrx2Δ cells. As shown in Fig. 2A, Gpx2 was oxidized with 0.1 mM H2O2 and at >0.4 mM H2O2, the proportion of Gpx2ox exceeded 40%. Regarding the redox of Gpx2 in t-BHP-treated cells, 0.2 mM t-BHP completely oxidized Gpx2 in trx1Δtrx2Δ cells; however, Gpx2 was mostly in the reduced form in wild-type cells even if 1.0 mM t-BHP was present. At lower concentrations of t-BHP (~0.5 μM), the proportion of Gpx2ox in trx1Δtrx2Δ cells was gradually increased in accordance with the concentrations of t-BHP (Fig. 2A, inset). We also monitored the time course of Gpx2 oxidation in trx1Δtrx2Δ cells after the treatment with 0.4 mM H2O2 or 0.6 mM t-BHP. In both cases Gpx2 was oxidized within 5 min after the treatment with these peroxides (Fig. 2B).

Gpx2 was completely oxidized after the treatment with 0.2 mM t-BHP for 5 min in trx1Δtrx2Δ cells; however, this was not the case in H2O2-treated cells even at higher concentrations and long-term exposure (Fig. 2). These results suggest that the prooxidant activity of t-BHP is higher than that of H2O2. In addition, because S. cerevisiae has two catalases (Ctt1 and Cta1), H2O2 may be decomposed in vivo. Furthermore, Collinson et al. (26) have reported that glutaredoxins (Grx1 and Grx2) exhibit GPx activity in the presence of GSH recycling system, and over-expression of either GRX1 or GRX2 conferred resistance to H2O2 but not to t-BHP (26). Taken together, H2O2 may be consumed more efficiently than t-BHP in trx1Δtrx2Δ cells, and therefore, complete oxidation of Gpx2 was not observed after H2O2 treatment.

Next, we investigated the effect of mitochondrial TRX-deficiency on the redox state of Gpx2. As shown in Fig. 1C, the ratio of Gpx2ox to total Gpx2 under non-stressed conditions slightly increased in a TRX-null mutant (trx1Δtrx2Δtrx3Δ) compared with that in the trx1Δtrx2Δ mutant. Intriguingly, Gpx2 was completely oxidized in trx1Δtrx2Δtrx3Δ cells after the treatment with 0.4 mM H2O2 (Fig. 1C), under which conditions ~50% of Gpx2 was in the reduced form in trx1Δtrx2Δ cells (Figs. 1, B and C). Gpx2 was mainly in the oxidized form in 0.6 mM t-BHP-treated trx1Δtrx2Δtrx3Δ cells, as was the case on trx1Δtrx2Δ cells (Figs. 1, B and C). These results suggest that the redox of Gpx2 is predominantly regulated by cytosolic TRX, although mitochondrial TRX also seems to contribute. It should be noted that the cytosolic TRX is a negative regulator of Yap1 (15), and GPX2 is one of the targets of this transcription factor (1, 18), therefore, the steady state level of Gpx2 increases in cells with the trx1Δtrx2Δ as well as trr1Δ background because of the constitutive activation of Yap1 (15, 27).

Redox State of Gpx2 Is Associated With That of TRX—The oxidized TRX is reduced by thioredoxin reductase. S. cerevisiae has two genes coding for this enzyme; i.e. TRR1 for reduction of the cytosolic TRX (Trx1 and Trx2), and TRR2 for mitochondrial TRX (Trx3). The Gpx2 in a trr1Δ mutant treated with 0.4 mM H2O2 was mostly in the reduced form (Fig. 1C), and this was also the case in a trr2Δ mutant (Fig. 1C). Meanwhile, Gpx2 was completely oxidized in trr1Δ cells after the treat-
ment with 0.6 mM t-BHP; nevertheless, Gpx2 was in the reduced form in trr2Δ cells under the same conditions (Fig. 1C). This suggests that the redox status of the cytosolic TRX may influence the redox state of Gpx2.

To explore the correlation between the redox state of TRX and that of Gpx2 more directly, we determined the redox state of TRX with an AMS modification assay. As shown in Fig. 3A, cytosolic TRX was virtually oxidized in WT and trrΔ cells after treatment with 0.4 mM H2O2 or 0.6 mM t-BHP. On the other hand, Gpx2 was in the reduced form in WT and trrΔ cells under the same conditions (Fig. 1C). This suggests that the redox status of the cytosolic TRX may influence the redox state of Gpx2.

FIGURE 3. Redox state of Gpx2 in vivo is linked with that of TRX. A, redox state of TRX in vivo. Cells (WT and trrΔ) were exposed to no chemical (lane 1), 0.4 mM H2O2 (lane 2), or 0.6 mM t-BHP (lane 3) for 1 h, and then TRX was modified with AMS. The percentage of TRXox in total TRX (sum of TRXred and TRXox) was plotted against the concentrations of peroxides. The density of each band in the Western blots was measured using densitometry. Open symbols, WT; closed symbols, trrΔ. B, kinetics of the Gpx2 oxidation after peroxide treatment. Cells (WT and trrΔ) were treated with 0.4 mM H2O2 or 0.6 mM t-BHP for different periods, and then Gpx2 was modified with AMS. The period of peroxide treatment in each lane was indicated in the figure. The percentage of Gpx2ox in total Gpx2 was plotted against the period of peroxide treatment. Open symbols, WT; closed symbols, trrΔ.

FIGURE 2. Effect of peroxides on the redox state of Gpx2 in vivo. A, effect of peroxide concentration. Cells (WT and trrΔ) were exposed to various concentrations of peroxides for 1 h as indicated in the figure, and subsequently, Gpx2 was modified with AMS. The percentage of Gpx2ox in total Gpx2 (sum of Gpx2red and Gpx2ox) was plotted against the concentrations of peroxides. The density of each band in the Western blots was measured using densitometry. Open symbols, WT; closed symbols, trrΔ. B, kinetics of the Gpx2 oxidation after peroxide treatment. Cells (WT and trrΔ) were treated with 0.4 mM H2O2 or 0.6 mM t-BHP for different periods, and then Gpx2 was modified with AMS. The period of peroxide treatment in each lane was indicated in the figure. The percentage of Gpx2ox in total Gpx2 was plotted against the period of peroxide treatment. Open symbols, WT; closed symbols, trrΔ.

Redox Regulation of Yeast Gpx2 by Thioredoxin
Redox Regulation of Yeast Gpx2 by Thioredoxin

FIGURE 4. Effect of Cys substitution on the redox state of Gpx2 in vivo. gpx2Δtrx1Δtrx2Δ cells carrying plasmid-borne Gpx2wt, Gpx2C10A, Gpx2C37A, Gpx2C65A, and Gpx2C83A were treated with 0.4 mM H2O2 or 0.6 mM t-BHP for 1 h, and the redox state of Gpx2 in each cell was determined.

FIGURE 5. Property of Gpx2 in vitro. A, purification of Gpx2. The His-tagged Gpx2 was expressed in E. coli, and samples at each purification step were subjected to reducing SDS-PAGE followed by staining with Coomassie Brilliant Blue. Samples in each lane are as follows: lane 1, cell extracts; lane 2, sample after HiTrap chelating column chromatography; lane 3, sample after thrombin treatment; lane 4, sample after HiTrap chelating column chromatography of thrombin-treated Gpx2. The apparent molecular mass of Gpx2 in reducing SDS-PAGE was slightly larger than that deduced from amino acid sequence (18 kDa) as previously reported (21). B, redox state of Gpx2 in vitro after treatment with H2O2 or t-BHP. Purified Gpx2 was incubated with various concentrations of H2O2, as indicated in the figure for 1 h and subjected to reducing and non-reducing SDS-PAGE. A small portion of Gpx2 formed a dimer through intermolecular disulfide bond.

ally in the reduced form in wild-type cells in the presence or absence of peroxides. On the other hand, small amounts of cytosolic TRX were in the oxidized form (TRXox) in trr1Δ cells under non-stressed conditions, and the proportion of TRXred increased after the treatment with 0.4 mM H2O2 (~50%) and reached 100% with 0.6 mM t-BHP. These results suggest that the redox state of Gpx2 is linked with that of TRX, i.e. Gpx2 in trr1Δ cells treated with H2O2 was in the reduced form because one-half of all the TRX was still TRXred under such conditions. Because the antibody against TRX we used is not able to distinguish between Trx1 and Trx2 (15), the TRX monitored here is the total cytosolic TRX.

Next, we expressed TRX1 or TRX2 with a multicopy plasmid in the trr1Δ mutant. We confirmed that the plasmid-borne Trx1 and Trx2 were functional in terms of the redox regulation of Gpx2 by expressing them in trr1Δ trr2Δ cells. As a result, the overexpression of TRX1 and TRX2 was not able to suppress the TRR1-deficiency with respect to the redox regulation of Gpx2 after the treatment with t-BHP (Fig. 3B). We also expressed GRX1 or GRX2 in the trr1Δtrr2Δ and trr1Δ mutants, although predictably, neither Grx1 nor Grx2 was able to compensate for the loss of TRX and its recycling system (Fig. 3C). Collectively, the redox state of Gpx2 in yeast cells is likely to be regulated in a TRX-dependent manner.

Intramolecular Disulfide Bond Formation between Cys37 and Cys83 of Gpx2—We could not detect the band shift corresponding to the dimer of Gpx2 in trr1Δtrr2Δ cells (Fig. 4). The reduced form of Gpx2 (Gpx2red) of the Cys-substituted Gpx2 mutants after the AMS modification migrated slightly faster than wild-type Gpx2red, which means that the number of AMS molecules associated with each Gpx2 molecule is decreased (WT, 4 × AMS; Cys mutants, 3 × AMS). The band corresponding to Gpx2red was not observed in C37A and C83A mutants after the treatment with H2O2 or t-BHP. This suggests that the intramolecular disulfide bond is formed between Cys37 and Cys83 (21). Consequently, the oxidized form of Gpx2 (Gpx2ox) of the C10A mutant after the AMS modification (Cys37-Cys83 and Cys65-AMS) migrated slightly faster than wild-type Gpx2ox (Cys37-Cys83 and Cys10-AMS), which was also because the number of AMS molecules able to associate with each Gpx2ox protein was decreased. However, intriguingly, the apparent molecular weight of the oxidized form of the C65A mutant (Cys37-Cys83 and Cys46-AMS) was almost the same as that of wild-type Gpx2ox; i.e. migration was slightly retarded. On the other hand, a faint band migrating slightly faster than the major reduced band appeared in the C83A mutant.

TRX-dependent Peroxidase Activity of Gpx2 in Vitro—We have demonstrated that the redox state of Gpx2 is regulated by a TRX-dependent system in vivo, which implies that Gpx2 behaves as a TPx. To address this possibility, we purified Gpx2 and measured the peroxidase activity with the TRX system (TRX, thioredoxin reductase, and NADPH) or with the GSH system (GSH, glutathione reductase, and NADPH).

His-tagged Gpx2 was purified from E. coli and the His tag was removed (Fig. 5A). The purified Gpx2 was subjected to a TPx assay. Gpx2 exhibited TPx activity toward H2O2 and t-BHP. This activity was dependent on both Gpx2 and TRX. The kinetic parameters are summarized in TABLE TWO. The peroxidase activity of Gpx2 toward H2O2 with the GSH system was ~10 times lower than that with the TRX system (TABLE TWO), and the catalytic efficiency of Gpx2 in the
Redox Regulation of Yeast Gpx2 by Thioredoxin

**TABLE TWO**

| Substrate | TRX system | GSH system |
|-----------|------------|------------|
|           | \( V_{\text{max}} \) | \( k_{\text{cat}} \) | \( K_m \) | \( V_{\text{max}} \) | \( k_{\text{cat}} \) | \( K_m \) |
| \( \text{H}_2\text{O}_2 \) | 2.60 \( \mu \text{mol/min/mg} \) | 9.57 \( \times 10^2 \) \( \mu \text{M} \) s\(^{-1} \) | 0.270 | 0.994 \( \times 10^2 \) \( \mu \text{mol/min/mg} \) | 313 | 1.09 \( \times 10^2 \) \( \mu \text{M} \) |
| \( \text{t-BHP} \) | 1.00 | 62.5 | 3.68 \( \times 10^2 \) | 0.295 | 170 | 0.994 \( \times 10^2 \) |

reduction of \( \text{H}_2\text{O}_2 \) with the TRX system was 100 times that with the GSH system \((k_{\text{cat}}/K_m)\) value in the TRX system was \(4.79 \times 10^6 \text{M}^{-1} \text{s}^{-1}\), and that in the GSH system was \(5.85 \times 10^5 \text{M}^{-1} \text{s}^{-1}\). On the other hand, the \( K_m \) value for \( \text{t-BHP} \) with the GSH system was 5 times larger than that with the TRX system, and the \( k_{\text{cat}}/K_m \) value for \( \text{t-BHP} \) with the TRX system was \(-10\) times larger \((5.89 \times 10^6 \text{M}^{-1} \text{s}^{-1})\) than that with the GSH system \((3.48 \times 10^5 \text{M}^{-1} \text{s}^{-1})\).

To confirm whether an intramolecular disulfide bond is formed in vivo, the enzyme was incubated with \( \text{H}_2\text{O}_2 \) without the TRX system. In this experiment, the \( \text{H}_2\text{O}_2 \)-treated Gpx2 was directly subjected to non-reducing and reducing SDS-PAGE, respectively, and the gels were stained for proteins with Coomassie Brilliant Blue. As shown in Fig. 5B, the majority of Gpx2 turned into the oxidized form after the treatment with \( \text{H}_2\text{O}_2 \), which migrates faster in non-reducing SDS-PAGE. Only a small portion of Gpx2 seems to form a dimer through an intermolecular disulfide bond. These results together with the redox state regulation with the TRX system, and the \( k_{\text{cat}}/K_m \) showed that a minute portion of Gpx2 seems to form a dimer through an intermolecular disulfide bond. These results together with the redox state regulation

### DISCUSSION

**Roles of Gpx2 as an Antioxidant—** *S. cerevisiae* has five TPX genes (TSA1, TSA2, AHP1, DOTS, and PRX1) (7), and the enzymatic properties of some of them have been extensively studied. The activity of Tsa1 toward \( \text{H}_2\text{O}_2 \) and \( \text{t-BHP} \) with the TRX system in vitro \((V_{\text{max}})\) values with \( \text{H}_2\text{O}_2 \) as substrate were \(4.8 \mu\text{mol/min/mg}\) of protein, and for \( \text{t-BHP}, 2.4 \mu\text{mol/min/mg}\) of protein \((28)\) was comparable with that of Gpx2 (TABLE TWO). However, because Tsa1 has higher affinity for peroxides \((K_m\) values for \( \text{H}_2\text{O}_2 \) were \(3 \mu\text{M}\) and for \( \text{t-BHP}\), were \(10 \mu\text{M}\)) than Gpx2 (TABLE TWO) and TSA1 is highly expressed throughout the growth phase of yeast \((29)\), Tsa1 is most likely to be the major TPX/Prx in the cytoplasm. On the other hand, a gpx3Δ mutant was hypersensitive to \( \text{H}_2\text{O}_2 \) and \( \text{t-BHP} \), whereas a gpx2Δ mutant was not \((1)\). Delaunay et al. (9) have reported that Gpx3 has TPx activity \((9)\). Although the \( V_{\text{max}} \) value of Gpx3 to \( \text{H}_2\text{O}_2 \) \((-1.29 \mu\text{mol/min/mg}\) of protein\) \((9)\) with the TRX system was lower than that of Gpx2 \((2.6 \mu\text{mol/min/mg}\) of protein\), the Gpx3 gene is constitutively expressed at higher levels in yeast cells \((1, 29)\). We then explored whether overexpression of Gpx2 can compensate for the loss of Gpx3 in vivo. As shown in Fig. 6C, susceptibility to peroxides of the gpx3Δ mutant was partially suppressed by overexpression of Gpx2.

We found that the viability of yeast cells was reduced when \( \text{CaCl}_2 \) and \( \text{t-BHP} \) coexisted, and this tendency was much more obvious in the gpx2Δ mutant \((Fig. 6A)\). As shown in Fig. 6B, \( \text{CaCl}_2 \) did not affect the redox state of Gpx2, whereas the amount of Gpx2 was further increased in cells treated with \( \text{CaCl}_2 \) and \( \text{t-BHP} \) concomitantly. We have previously reported that oxidative stress-induced expression of Gpx2 is independent of the regulatory mechanism of \( \text{Ca}^{2+} \)-mediated signaling to the Gpx2 promoter \((16)\), and therefore, an additive effect in terms of the induction of Gpx2 in the presence of peroxide and \( \text{CaCl}_2 \) was observed in the Gpx2-lacZ reporter assay \((16)\). We confirmed that this is true at the protein level \((Fig. 6B)\). Taken together, our results suggest that Gpx2 is important in the oxidative stress-induced response in the presence of \( \text{Ca}^{2+} \).

In mammalian cells excessive \( \text{Ca}^{2+} \) accumulation in mitochondria induces opening of the mitochondrial permeability transition pore, which eventually leads to the release of cytochrome c from mitochondria, an early trigger in the apoptotic cascade \((30)\). By contrast, in *S. cerevisiae*, \( \text{Ca}^{2+} \) alone is not able to raise the permeability of the mitochondrial membrane, although the co-existence of \( \text{t-BHP} \) leads to the similar phenomenon of opening of mitochondrial permeability transition pore \((31)\). In mammals, the opening of mitochondrial permeability transition pore is inhibited by many antioxidants \((32–36)\), and the same thing can be observed in yeast mitochondria; i.e. Kowaltowski et al. \((31)\) reported that Tsa1 and catalase protect mitochondria from permeabilization induced by \( \text{Ca}^{2+} \) and \( \text{t-BHP} \). We have not yet determined whether or not the cell death caused by \( \text{t-BHP} \) and \( \text{Ca}^{2+} \) is apoptosis; determination of the physiological relevance as well as intracellular localization of Gpx2 in the response to oxidative stress in the presence of \( \text{Ca}^{2+} \) is now underway.

**Redox Regulation of Non-SeCys-type Gpx—** Rocher et al. \((3)\) reported that the substitution of SeCys with Cys in murine cytosolic Gpx \((\text{cGpx/Gpx1})\) decreased the enzyme activity 1000-fold. This was also the case for PHGpX \((4)\). In the peroxidase reaction of Prxs, the conserved per-
oxidative cysteine (Cys-SH) is oxidized to cysteine sulfenic acid (Cys-SPOH). Therefore, Cys at the active site of the SeCys-substituted GPx is presumed to be oxidized to Cys-SOH by H2O2. cGPx/GPx1 is a homotetramer, and PHGPx is a monomeric enzyme. If a disulfide bond is formed intermolecularly or intramolecularly as a consequence of the reduction of H2O2 by the SeCys-substituted enzyme, a thiol-disulfide oxidoreductase may be necessary for recovery of the active site Cys. The same thing may be applied also to the peroxidase reaction carried out by the "GPx-like proteins" that do not contain SeCys at the active site. We have demonstrated here that an intramolecular disulfide bond between Cys37 and Cys83 seems to be formed within a monomer of Gpx2 in vivo (Fig. 4). Presumably, Cys37 may function as a peroxidatic Cys, and Cys83 may be a resolving Cys analogous to Gpx3.

Regarding the reduction of Gpx2, TRX, but not glutaredoxin, is the preferable reductant to recover Cys37-SH and Cys83-SH in vivo, and GSH is also able to function as a reducing power in vitro even if the efficiency is lower than that by TRX. Delaunay et al. (9) have reported that the peroxidase activity of Gpx3 is strictly dependent on TRX, and GSH is not able to serve as the reducing power for Gpx3 in vitro. However, Avery and Avery (2) have reported that Gpx3 showed GSH-dependent peroxidase activity toward t-BHP and phospholipid hydroperoxide. Hence, the electron donor for Gpx3 is still controversial.

**FIGURE 6. Investigation of the physiological role of Gpx2.** A, susceptibility of yeast cells to t-BHP and Ca2+. Cells (WT and gpx2Δ) were cultured in YPD medium until A610 = 0.5 and treated with 3 mM t-BHP, 200 mM CaCl2, or both for the prescribed period as indicated in the figure, and viability was determined. B, effect of CaCl2 on the redox state of Gpx2. Cells (WT, trx1Δtrx2Δ, and trr1Δ) were cultured in YPD medium until A610 = 1 and treated with 0.6 mM t-BHP (lane 2), 200 mM CaCl2 (lane 3), or 200 mM CaCl2 and 0.6 mM t-BHP (lane 4) for 1 h, and the redox state of Gpx2 in each cell was determined. Lane 1, control. C, effect of GPX2 overexpression in gpx3Δ cells on suppression of peroxide sensitivity. gpx3Δ cells carrying pRS413 (vector) or pRSGPX2–3H (GPX2–3) were cultured until A610 = 0.5, and the Gpx2 protein level was determined by Western blotting (upper panel). Because Yap1 is not fully activated in gpx3Δ cells, induction of GPX2 expression was not observed (18). To determine the susceptibility to peroxides, gpx3Δ cells carrying pRS413 (vector) or pRSGPX2–3H (GPX2–3) were serially diluted and spotted onto SD agar plates containing H2O2 or t-BHP.
As a result of alanine scanning of Cys residues of Gpx2, the C65A mutant seems to be tolerant to peroxides in terms of the formation of a disulfide bond, i.e. a portion of wild-type Gpx2 as well as the C10A mutant was oxidized in \( \text{trXR} \Delta \text{trx2A} \) cells without peroxides, whereas the C65A mutant was substantially in the reduced form (Fig. 4). This was also the case in \( H_2O_2 \)-treated cells. A large part of Gpx2 of wild-type and mutant was oxidized in \( H_2O_2 \) treatment with \( t-BHP \), although approximately one-half of the C65A mutant was still in the reduced form under the same conditions, suggesting that Cys\(^{65} \) influences the disulfide bond formation between Cys\(^{37} \) and Cys\(^{83} \) of Gpx2 by a mechanism yet to be determined.

**Phylogenetic Position of Gpx2 as an Atypical 2-Cys Peroxiredoxin**

Mammalian GPxs are classified into four groups, *i.e.* classical cellular and cytoplasmic GPx (cGPx/GPx1), gastrointestinal GPx (giGPx/GPx2), extracellular plasma GPx (pGPx/GPx3), and phospholipid hydroperoxide GPx (PHGPx/GPx4). These GPxs contain SeCys at the active site (SeCys-type GPx) and constitute the catalytic triad with two amino acids that constitute the GSH binding site was conserved in yeast homologues of mammalian GPx (1), although, intriguingly, none of the amino acids that constitute the GSH binding site was conserved; however, these amino acids were poorly conserved in PHGPxs. This may partially explain the fact that PHGPx requires high concentrations of GSH for the reduction of phospholipid hydroperoxide in *vitro* (37). We have cloned \( \text{GPX1} \), \( \text{GPX2} \), and \( \text{GPX3} \) as structural homologues of mammalian GPx (1), although, intriguingly, none of the amino acids that constitute the GSH binding site was conserved in yeast homologues (1). This may in part account for the difference in preference of the electron donor for the peroxidase activity between mammalian GPxs, including PHGPx and yeast "GPx homologues."

Besides *S. cerevisiae* GPx homologues, many GPx-like proteins that do not contain SeCys (non-SeCys-type GPx) have been found in several biological sources. For example, the fission yeast *Schizosaccharomyces pombe* has a \( gpx1^+ \) gene (38). Mice have the epididymis-specific secreting GPx (eGPx/GPx5), and tomato (*Lycopersicon esculentum*) showed extremely lower peroxidase activity when GSH was used as the electron donor (41, 42). By contrast, these enzymes have TRX-dependent peroxidase activity (40, 42). Although the redox regulation of many other GPx-like proteins in plants has not yet been determined, the Chinese cabbage GPx homologue has been reported to make intramolecular disulfide bond (atypical 2-Cys Prx-type redox regulation) as a consequence of the peroxidase reaction (40). Taken together, the non-SeCys-type PHGPx homologues may constitute a family of atypical 2-Cys Prxs.

**REFERENCES**

1. Inoue, Y., Matsuda, T., Sugiyama, K., Izawa, S., and Kimura, A. (1999) *J. Biol. Chem.* 274, 27002–27009
2. Avery, A. M., and Avery, S. V. (2001) *J. Biol. Chem.* 276, 33730–33735
3. Rocher, C., Lalanne, J. L., and Chaudiere, J. (1992) *Eur. J. Biochem.* 205, 955–960
4. Maiorino, M., Aumann, K. D., Brigelius-Flohe, R., Doria, D., van den Heuvel, J., McCarthy, J., Roveri, A., Ursini, F., and Flohe, L. (1995) *Biochim. Biophys. Acta* 1276, 651–660
5. Hofmann, B., Hecht, H. J., and Flohe, L. (2002) *Biochem. Biophys. Res. Commun.* 293, 347–346
6. Molaire, X., Krimm, I., Ebel, C., Verdoucq, L., Prouzet-Mauleon, V., Chartier, Y., Tzan, P., Lassquin, G., Meyer, Y., and Lancelin, J.-M. (2003) *Biochemistry* 42, 14139–14149
7. Park, S. G., Cha, M. K., Jeong, W., and Kim, I. H. (2000) *J. Biol. Chem.* 275, 5723–5732
8. Pedrojas, J. R., Miranda-Vizuete, A., Jamvandary, N., Gustafsson, J.-Å., and Spyrou, G. (2000) *J. Biol. Chem.* 275, 16296–16301
9. Delaunay, A., Pfieger, D., Barrault, M. B., Vinh, L., and Toledano, M. B. (2002) *Cell* 111, 471–481
10. Moye-Rowley, W. S. (2003) *Eukaryot. Cell* 2, 381–389
11. Kuge, S., Jones, N., and Nomoto, A. (1999) *EMBO J.* 18, 1710–1720
12. Coleman, S. T., Epping, E. A., Steggerda, S. M., and Moye-Rowley, W. S. (1999) *Mol. Biol. Cell* 10, 8302–8313
13. Delaunay, A., Isnard, A. D., and Toledano, M. B. (2000) *EMBO J.* 19, 5157–5166
14. Pearson, G. D., and Merrill, G. F. (1998) *J. Biol. Chem.* 273, 5431–5434
15. Izawa, S., Maeda, K., Sugiyama, K., Mano, J., Inoue, Y., and Kimura, A. (1999) *J. Biol. Chem.* 274, 28459–28465
16. Tsuzi, D., Maeta, K., Takatsune, Y., Izawa, S., and Inoue, Y. (2004) *FEBS Lett.* 569,
17. Frand, A. R., and Kaiser, C. A. (2000) Mod. Biol. Cell 11, 2833–2843
18. Tsuzi, D., Maeta, K., Takatsuse, Y., Izawa, S., and Inoue, Y. (2004) FEBS Lett. 565, 148–154
19. Ursini, F., Heim, S., Kiess, M., Maiorino, M., Roveri, A., Wissing, J., and Flohe, L. (1999) Science 285, 1393–1396
20. Collinson, L. P., and Dawes, I. W. (1995) Gene (Amst.) 156, 123–127
21. Izawa, S., Maeda, K., Miki, T., Mano, J., Inoue, Y., and Kimura, A. (1998) Biochem. J. 330, 811–817
22. Bjornstedt, M., Xue, J., Huang, W., Akesson, B., and Holmgren, A. (1994) J. Biol. Chem. 269, 29382–29384
23. Takebe, G., Yarimizu, J., Saito, Y., Hayashi, T., Nakamura, H., Yodoi, J., Nagaosawa, S., and Takahashi, K. (2002) J. Biol. Chem. 277, 41254–41258
24. Gan, Z. R. (1991) J. Biol. Chem. 266, 1692–1696
25. Pedrajas, J. R., Kosmidou, E., Miranda-Vizuete, A., Gustafsson, J. A., Wright, A. P., and Spyrou, G. (1999) J. Biol. Chem. 274, 6366–6373
26. Collinson, E. J., Wheeler, G. L., Garrido, E. O., Avery, A. M., Avery, S. V., and Grant, C. M. (2002) J. Biol. Chem. 277, 16712–16717
27. Carmel-Harel O., Stearman, R., Gasch, A. P., Botstein, D., Brown, P. O., and Storz, G. (2001) Mol. Microbiol. 39, 595–605
28. Jeong, J. S., Kwon, S. J., Kang, S. W., Rhee, S. G., and Kim, K. (1999) Biochemistry 38, 776–783
29. Monje-Casas, F., Michan, C., and Pueyo, C. (2004) Biochem. J. 383, 139–147
30. Bernardi, P. (1999) Physiol. Rev. 79, 1127–1155
31. Kowaltowski, A. J., Vercesi, A. E., Rhee, S. G., and Netto, L. E. (2000) FEBS Lett. 473, 177–182
32. Valle, V. G., Fagian, M. M., Parentoni, L. S., Meinicke, A. R., and Vercesi, A. E. (1993) Arch. Biochem. Biophys. 307, 1–7
33. Castilho, R. F., Kowaltowski, A. J., Meinicke, A. R., Bechara, E. J., and Vercesi, A. E. (1995) Free Radic. Biol. Med. 18, 479–486
34. Kowaltowski, A. J., Castilho, R. F., Grijalba, M. T., Bechara, E. J., and Vercesi, A. E. (1996) J. Biol. Chem. 271, 2929–2934
35. Kowaltowski, A. J., Castilho, R. F., and Vercesi, A. E. (1996) FEBS Lett. 378, 150–152
36. Kowaltowski, A. J., Netto, L. E., and Vercesi, A. E. (1998) J. Biol. Chem. 273, 12766–12769
37. Ursini, F., Maiorino, M., Brigelius-Flohe, R., Aumann, K. D., Roveri, A., Schomburg, D., and Flohe, L. (1995) Methods Enzymol. 252, 38–53
38. Yamada, K., Nakagawa, C. W., and Mutoh, N. (1999) Yeast 15, 1125–1132
39. Vernet, P., Rigaudiere, N., Ghyselinck, N., Dufaure, J. P., and Drevet, J. R. (1996) Biochem. Cell Biol. 74, 125–131
40. Jung, B. G., Lee, K. O., Lee, S. S., Chi, Y. H., Jang, H. H., Kang, S. S., Lee, K., Lim, D., Yoon, S. C., Yoon, D. J., Inoue, Y., Cho, M. J., and Lee, S. Y. (2002) J. Biol. Chem. 277, 12572–12578
41. Holland, D., Ben-Hayyim, G., Faltin, Z., Camoin, L., Strosberg, A. D., and Eshdat, Y. (1993) Plant Mol. Biol. 21, 923–927
42. Herbette, S., Lenne, C., Leblanc, N., Julien, J. L., Drevet, J. R., and Roeckel-Drevet, P. (2002) Eur. J. Biochem. 269, 2414–2420