Purification and Characterization of a Second Type Thioredoxin Peroxidase (Type II TPx) from *Saccharomyces cerevisiae*†

Jin Sook Jeong,‡ Soo Jin Kwon,‡ Sang Won Kang,§ Sue Goo Rhee,§ and Kanghwa Kim*,‡

Department of Food and Nutrition, College of Home Economics, Chonnam National University, Korea, and Laboratory of Cell Signaling, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892

Received July 23, 1998; Revised Manuscript Received October 28, 1998

ABSTRACT: A yeast peroxidase that reduces H_2O_2 and alkyl hydroperoxides with the use of reducing equivalents provided by thioredoxin was identified previously and named thioredoxin peroxidase (TPx) [Chae, H. Z., Chung, S. J., and Rhee, S. G. (1994) *J. Biol. Chem. 269*, 27670−27678]. A second type thioredoxin-dependent peroxidase, named type II TPx, has now been purified from yeast, and several peptide sequences have been obtained. Using those sequences, the corresponding cDNA has been identified from the GenBank database. Comparison of the predicted sequence of 176 amino acids of type II TPx with that of the 195 residues of TPx, now renamed type I TPx, revealed no substantial homology except for a short segment preceding Cys^{62} of type II TPx. Kinetic characterization of the reactions catalyzed by type I and II TPxs revealed that type I preferentially reduces H_2O_2 rather than alkyl hydroperoxides, whereas type II shows the reverse specificity. Type II TPx contains three cysteine residues at positions 31, 62, and 120. Experiments with mutant proteins in which these three cysteine residues were replaced individually with serine suggest that Cys^{62}—SH constitutes the site of oxidation by peroxides and that the oxidized Cys^{62} reacts with the Cys^{120}—SH group of another type II TPx molecule to form an intermolecular disulfide linkage. The formed disulfide can then be reduced by thioredoxin, but not by glutathione. Thus, type II TPx mutants lacking Cys^{62} or Cys^{120} showed no detectable TPx activity, whereas mutation of Cys^{31} had no effect on TPx activity. An antioxidant function of type II TPx in intact cells was demonstrated by the observation that *Escherichia coli* cells overexpressing wild-type protein were less sensitive to inhibition of growth by alkyl hydroperoxides than were control cells or cells overexpressing the mutant protein lacking Cys^{62}.

In the presence of Fe^{3+} and an electron donor such as dithiothreitol (DTT)[1] or ascorbate, O_2 is reduced to $O_2^{\bullet-}$, which disproportionate rapidly to O_2 and H_2O_2. Hydrogen peroxide, in turn, can be reduced further, via the Fenton reaction, to $OH^{\bullet}$ and OH^-. The resulting hydroxyl radicals are extremely reactive and can damage a variety of cellular components, including the enzyme glutamine synthetase (*1*, *2*). Crude extracts of *Saccharomyces cerevisiae* were previously shown to contain a factor that confers protection against damage by the thiol oxidation system (Fe^{3+}, O_2, and either DTT or 2-mercaptoethanol) by a mechanism that is distinct from those of conventional antioxidant enzymes such as catalase, superoxide dismutase, and glutathione peroxidase (*3*). The protective factor was a 25-kDa protein (*3*).

The purified 25-kDa protein protects various enzymes from the effects of the thiol oxidation system but not from those of the ascorbate oxidation system (Fe^{3+}, O_2, and ascorbate) (*3*). For this and other reasons described previously (*4*), the 25-kDa enzyme was named thiol-specific antioxidant (TSA) (*5*−*9*). However, the antioxidant activity of TSA was subsequently shown to be attributable to its ability to reduce H_2O_2 (*10*). The apparent specific requirement of a thiol for antioxidant function was because an intermolecular disulfide linkage of oxidized TSA can be reduced by a thiol but not by ascorbate (*10*). We have shown that the physiological electron donor for the reduction of TSA is thioredoxin (Trx), which ultimately receives electrons from NADPH via Trx reductase (TR) (*10*). TSA was thus the first peroxidase to be identified for which Trx is the immediate electron donor, and it was therefore renamed Trx peroxidase (TPx). The yeast TPx (TSA) gene has been cloned and sequenced (*5*, *6*), and the predicted protein shows no substantial homology to known catalase, superoxide dismutase, or peroxidase enzymes.

The 25-kDa TPx (now renamed type I TPx for the reason described below) was purified from yeast homogenate on the basis of its ability to protect glutamine synthetase from inactivation by the thiol oxidation system (*3*). The inactivation is thought to be caused by $OH^{\bullet}$ derived from H_2O_2 through the Fenton reaction (*2*, *3*). Therefore, the glutamine synthetase protection assay is an indirect method that does

† This research was supported partly by the Korea Research Foundation (to K.K.).

* To whom correspondence should be addressed at Department of Food and Nutrition, College of Home Economics, Chonnam National University, Kwang-Ju, 500-757, Korea. Tel: 82-62-530-1334. Fax: 82-62-530-1339.

‡ Chonnam National University.

§ National Heart, Lung, and Blood Institute, National Institutes of Health.

[1] Abbreviations: DTT, dithiothreitol; TSA, thiol-specific antioxidant; Trx, thioredoxin; TR, Trx reductase; TPx, Trx peroxidase; PCR, polymerase chain reaction; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid; HPLC, high-performance liquid chromatography; TNB, 2-nitro-5-thiobenzoic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Prx, peroxiredoxin.

10.1021/bi9817818 CCC: $18.00
Published on Web 12/19/1998

not measure peroxidase activity quantitatively. While purifying type I TPx, we noticed a minor peak of glutamine synthetase protection activity that was well separated from the type I TPx peak. We repeated the chromatographic fractionation of yeast proteins and assayed the fractions spectrophotometrically by monitoring H_2O_2-dependent NADPH oxidation in the presence of Trx and TR. A peak more prominent than that for type I TPx was observed where the minor peak of glutamine synthetase protection activity was detected previously. We now describe the purification to homogeneity of this new thioredoxin peroxidase, named type II TPx, as well as the identification and expression of the corresponding yeast cDNA. We have also compared the catalytic properties of type I and II TPxs, and two cysteine residues that are essential for the catalytic activity of type II TPx were identified.

EXPERIMENTAL PROCEDURES

Materials. Type I TPx and TR were purified to homogeneity from *S. cerevisiae* as described (*3*, *10*). *Escherichia coli* glutamine synthetase was isolated as described (*12*). Cumene hydroperoxide was obtained from ICN and *tert*-butyl hydroperoxide was from Sigma.

Purification of Trx1 and Trx2. Yeast contains two forms of thioredoxin, Trx1 and Trx2, and their cDNAs have been cloned (*13*, *14*). These cDNAs were obtained by polymerase chain reaction (PCR) with yeast genomic DNA (Clontech) as template. The forward primers for Trx1 (5′-ATCAT*ATG*-GTTACTCAATTCAAAACTGC-3′) and Trx2 (5′-ATCAT-*ATG*GTCACTCAATTAAAATCCGCTTC-3′) contain an *Nde*I site (underlined) and the initiation codon (italicized). The reverse primers for Trx1 (3′-CGTTCGGTAACGAC-GATTACGA*ATT*GATCAA-5′) and Trx2 (3′-CGATAAC-GAAGGTTGCAT*ATC*TGATCAA-5′) contain an *Spe*I site (underlined) and the stop codon (italicized). The PCR products were purified and cloned into the pCRII vector (Invitrogen). The resulting pCRII constructs were digested with *Nde*I and *Spe*I, and the *Nde*I−*Spe*I fragment containing the coding region was ligated into the pET17b vector (Novagen). *E. coli* BL21cells were transformed separately with each of the expression vectors, and colonies containing each vector were grown at 37 °C. Expression of Trx proteins was then induced for 6 h at 30 °C with 0.1 mM isopropyl-β-D-thiogalactopyranoside. Trx1 and Trx2 were purified by heat treatment at 55 °C for 2 min, ammonium sulfate precipitation at 95% saturation, and sequential chromatographies on Sephacryl S-200 gel filtration column and HPLC Mono Q column.

Assay of Column Fractions for TPx Activity. NADPH oxidation was monitored as A_{340} in a 0.5-mL reaction mixture containing 50 mM Hepes-NaOH (pH 7.0), 0.2 mM NADPH, 10 μM Trx, 0.3 μM TR, 1 mM H_2O_2, and a portion of column chromatography fractions. The reaction was started by addition of 50 μL of peroxide solution, and the mixture was incubated at 30 °C. One unit of peroxidase activity corresponds to the oxidation of 1 mmol of NADPH per minute.

Glutamine Synthetase Protection Assay. Glutamine synthetase inactivation was performed in a 25-μL reaction mixture containing 50 mM Hepes-NaOH (pH 7.0), 1 μg of glutamine synthetase, 10 mM DTT, 3 μM $FeCl_3$, and various concentrations of type II TPx. After 10 min at 30 °C, the remaining glutamine synthetase activity was measured by the γ-glutamyltransferase method (*3*).

Purification of Type II TPx. Frozen *S. cerevisiae* BJ986 cells (300 g) were suspended in 1.2 L of 20 mM Hepes-NaOH (pH 7.0) containing 2 mM phenylmethylsulfonyl fluoride and homogenized in a Bead Beater (Biospec Products, Bartlesville, OK) as described (*3*). The cell extract was centrifuged for 30 min at 9000*g*, and the resulting supernatant was adjusted to 1% (w/v) streptomycin sulfate by addition of a 10% solution. After incubation for 15 min at 4 °C, the suspension was centrifuged at 45 000*g* for 30 min to remove the nucleic acid precipitate. The resulting supernatant was adjusted to 12% (w/v) poly(ethylene glycol) (8000 Da) by the addition of a 40% solution. After incubation for 15 min at 4 °C, the suspension was centrifuged at 4000*g* for 10 min, and the resulting pellet was resuspended in 160 mL of 50 mM Tris-HCl (pH 7.6). Insoluble material remaining after resuspension was removed by centrifugation at 45 000*g* for 40 min.

The final supernatant (8.2 g of protein) was applied to a DEAE-Sephacel (Pharmacia) column (2.5 × 50 cm) that had been equilibrated with 50 mM Tris-HCl (pH 7.6). The column was washed with 400 mL of equilibration buffer and proteins were eluted with a linear NaCl gradient from 0 to 0.4 M in 4 L of 50 mM Tris-HCl (pH 7.6). Fractions of 20 mL were collected. Peak fractions of peroxidase activity (fractions 124−138) were pooled, and the proteins were precipitated with 70% saturated ammonium sulfate. The resulting precipitate was dissolved in 150 mL of 20 mM Hepes-NaOH (pH 7.0) containing 0.5 M ammonium sulfate, and insoluble material was removed by centrifugation. The resulting supernatant was divided into four equal portions, which were further fractionated independently. Each portion (210 mg of protein) was applied to a TSK-gel (Tosohaas) preparative phenyl-5PW column (21.5 × 150 mm) that had been equilibrated with 20 mM Hepes (pH 7.0) containing 1 M ammonium sulfate. Proteins were eluted from the column at a flow rate of 5 mL/min by reducing the ammonium sulfate concentration to zero for 60 min, and fractions of 5 mL were collected. Two peaks of peroxidase activity, centered at fractions 27 and 40, were observed. The later and smaller peak was shown to contain type I TPx by immunoblot analysis. Fractions corresponding to the larger peak (fractions 26−30) from all four chromatographic separations were pooled, concentrated in a Centriprep 30 filtration cell (Amicon), and washed with 50 mM Tris-HCl (pH 7.6) in the same device. Each (55 mg of protein) of the halves of the resulting sample was applied independently to an HPLC Mono Q column (10 × 100 mm) that had been equilibrated with 50 mM Tris-HCl (pH 7.6). Proteins were eluted at a flow rate of 2 mL/min with a linear gradient of NaCl from 0 to 0.4 M during 40 min, and fractions of 2 mL were collected. Peak fractions (fractions 29−34) from the two chromatographic separations were pooled and concentrated, and each (16 mg of protein) of the halves of the concentrated sample was applied independently to a TSK-gel HA-1000 hydroxyapatite column (7.5 × 75 mm) that had been equilibrated with 5 mM sodium phosphate buffer (pH 7.2). Proteins were eluted at a flow rate of 1 mL/min with a linear gradient of sodium phosphate from 5 to 100 mM, and fractions of 1 mL were collected. Peak fractions (fractions

12−18) from the two chromatographic separations were pooled, concentrated, and stored in aliquots at −70 °C.

Tryptic Peptides of Type II TPx. Purified type II TPx (0.2 mg) was reductively denatured with 50 mM Tris-HCl (pH 8.0) containing 6 M guanidine hydrochloride and 2 mM DTT. The sulfhydryl groups were labeled with 2-nitro-5-thiobenzoic acid (TNB) by adding Ellman's reagent, 5′,5′-dithiobis-(2-nitrobenzoic acid), to a final concentration of 10 mM. The TNB-conjugated protein was precipitated with trichloroacetic acid, washed with acetone, suspended in 100 μL of 100 mM Tris-HCl (pH 8.0), and digested with trypsin overnight at 30 °C. A portion of the generated peptides were applied to a Vydac C_{18} column (4.6 × 250 mm) and eluted with a linear gradient of acetonitrile from 0 to 60% (v/v) in 0.1% trifluoroacetic acid during 60 min. Six peptides indicated in Figure 2 were subjected to amino acid sequence analysis. Another portion of the tryptic peptides was treated with DTT to remove the TNB group before separation.

Cloning and Expression of the Type II TPx Gene. A full-length type II TPx cDNA was amplified from the yeast genomic library (Clontech) by PCR with the forward primer 5′-ATCAT*ATG*TCTGACTTAGTTAACAAGAA-3′, which contains an *Nde*I site (underlined) and the initiation codon (italicized), and the reverse primer 3′-CACAGAACCGAG-TAAAC*AT*CTTAAGTT-5′, which contains an *Eco*RI site (underlined) and the stop codon (italicized). The resulting PCR product was used for the expression of type II TPx proteins in *E. coli* as described above for the production of Trx proteins. Three mutant type II TPx proteins, C31S, C62S, and C120S, in which Cys^{31}, Cys^{62}, and Cys^{120} were replaced individually by serine, were generated by standard PCR-mediated site-directed mutagenesis with complementary primers containing a 1-bp mismatch that converts the codon for cysteine to one for serine. The recombinant proteins were isolated by a procedure similar to that described above for purification of the native protein from *S. cerevisiae* and the purity of all the purified proteins was confirmed as electrophoretically homogeneous by SDS−PAGE.

Determination of Protein Concentration. The concentrations of type I TPx, type II TPx, Trx1, and Trx2 were determined spectrophotometrically with absorptivity ($A^{0.1\%}$) at 280-nm values of 1.1, 1.6, 0.98, and 0.86, respectively; these values were calculated on the basis of the amino acid composition of the proteins deduced from the corresponding cDNA sequences. The concentration of TR was determined with the Bicinchoninic acid protein assay reagent (Pierce), with bovine serum albumin as standard.

Antibodies to Type II TPx. Antiserum specific to type II TPx was prepared by immunizing rabbits with type II TPx purified from *S. cerevisiae*.

RESULTS

Purification of Type II TPx. Fractionation of yeast extract on a DEAE-Sephacel column yielded a single peak, centered at fraction 130, of peroxidase activity monitored as the H_2O_2-dependent oxidation of NADPH in the presence of Trx and TR (Figure 1A). Subsequent purification of pooled fractions 124 to138 on an HPLC phenyl-5PW column yielded two peaks of activity centered at fractions 27 and 40 (Figure 1B). Immunoblot analysis with antibodies to type I TPx revealed that the second peak of activity was attributable to type I

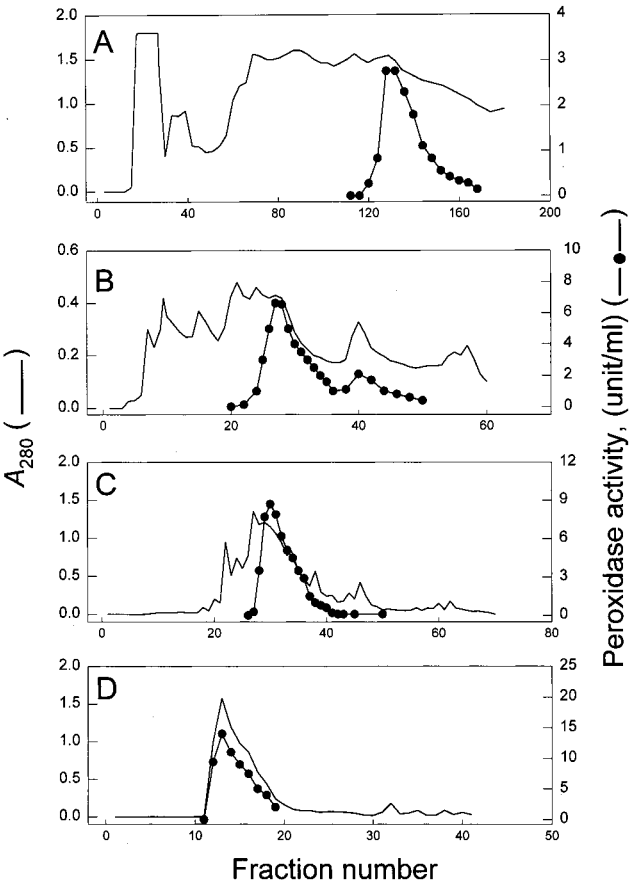

FIGURE 1: Purification of yeast TPx2. *S. cerevisiae* proteins (8.2 g) were subjected to chromatography on DEAE-Sephacel (A), HPLC TSK phenyl-5PW (B), HPLC Mono Q (C), and HPLC TSK-gel HA-1000 hydroxyapatite (D) columns as described in Experimental Procedures.

TPx, whereas the first peak contained no detectable type I TPx. Further purification of fractions corresponding to the first peak on an HPLC Mono Q column (Figure 1C) followed by an HPLC hydroxyapatite column (Figure 1D) yielded an activity peak with a shoulder that coincided with the protein profile measured as A_{280}. Analysis of peak fractions by SDS−PAGE revealed a protein with an apparent molecular mass of 22 kDa, which we named type II TPx. We obtained 16 mg of type II TPx from 300 g (wet weight) of yeast.

Cloning of the Type II TPx Gene. Purified type II TPx was labeled with TNB and digested with trypsin, and the resulting peptides were fractionated on a C_{18} column. The six peptides (peptides 1−6) indicated in Figure 2 were subjected to sequence analysis. Peptides 2, 3, and 6 were included because they were judged to contain TNB-modified cysteine residues on the basis of their UV spectra and the observation that their elution times from the C_{18} column were reduced by 1 min after treatment with DTT (data not shown). None of the sequences of peptides 1 to 5 showed homology to type I TPx. However, peptide 6 (VIITGAPAAFSPTCTV-SHIP) showed low sequence homology to amino acids 34 to 53 of type I TPx (VVLAFIPLAFTFVCPTEIIA) (see Figure 3).

A search of the *S. cerevisiae* genome database revealed that all six peptide sequences were contained in an open reading frame encoding 176 amino acids (EMBL accession number z73281; chromosome XII). The calculated molecular mass of type II TPx is 19 114 Da. A comparison of the

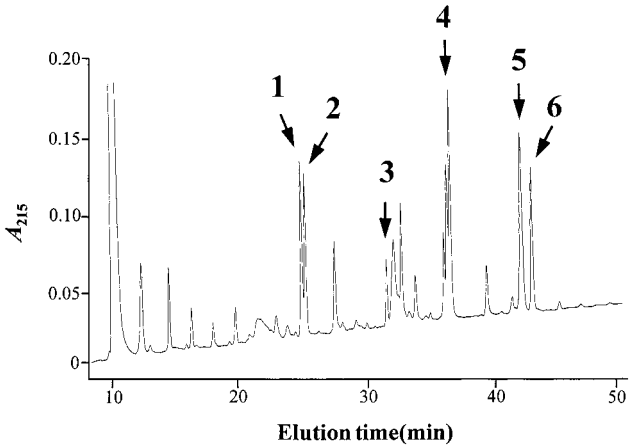

FIGURE 2: Isolation and sequences of tryptic peptides of type II TPx. Purified yeast type II TPx (0.2 mg) was labeled with TNB and then subjected to digestion with trypsin. The resulting peptides were separated on a C_{18} column. Peptides that were subjected to sequence analysis are indicated by numbers. Peptides 1, MPQTVEWSK; 2, FASDPGCAFT; 3, FQYIATSQSDADSESCK-MPQ; 4, WAMVVENGI; 5, EVDQVEVVTVDNPFA; 6, VIIT-GAPAAFSPTCTVVHIP.

deduced amino acid sequence with that of type I TPx using the GCG PILEUP program revealed no significant overall similarity. Because Cys^{47} of type I TPx is the site of the peroxidase reaction (*7*, *10*) and the sequence of a short segment preceding Cys^{47} is conserved in all members of TPx homologues named Prx proteins (*6*), we visually scanned type II TPx for a region homologous to this Cys^{47}-containing segment and found a noticeable match to a short region preceding Cys^{62} (Figure 3). *S. cerevisiae* chromosome IV (GenBank accession number U33007) contains an open reading frame encoding a 195-residue protein that shows 86.1% sequence identity (93.8% similarity) to type I TPx. This type I homologue is probably a TPx and shows no sequence similarity to type II TPx except in the short sequence preceding the conserved cysteine (Figure 3).

Catalytic Properties of Type II TPx. The rate of H_2O_2 reduction catalyzed by purified yeast type II TPx was measured by monitoring the decrease in A_{340} attributable to the oxidation of NADPH (Figure 4). The oxidation of NADPH required all three protein components (type II TPx, Trx, and TR), being negligible in the absence of any one. Thus, Trx and TR, despite their redox-sensitive cysteine residues, cannot reduce H_2O_2 to a significant extent in the absence of type II TPx. Similar results were obtained when the reduction of H_2O_2 was measured directly with the use of ferrithiocyanate (*15*) (data not shown). We also investigated whether glutathione can provide reducing equivalents for the reduction of H_2O_2 by monitoring NADPH oxidation with a reaction mixture containing H_2O_2, type II TPx, glutathione, glutathione reductase, and NADPH. No NADPH oxidation was apparent under these conditions (data not shown).

Type II TPx was expressed in *E. coli* and purified from the soluble fraction of the bacterial cells to yield a preparation with a purity of >95% (not shown). The catalytic activity of the recombinant enzyme, as measured by the reduction of H_2O_2, was virtually identical with that of the native protein purified from yeast (data not shown). We next measured the initial rates of NADPH oxidation catalyzed by purified type I and II TPxs in the presence of various concentrations of the two isoforms of Trx. Lineweaver−Burk plots (not shown) revealed that the K_m values for both Trx1 and Trx2 were between 2 and 3 μM for the reactions catalyzed by either type I or II TPx. The V_{max} values at 30 °C were 16 to 17 and 5.5 μmol min^{-1} mg^{-1} of protein for type II and I TPx, respectively, regardless of whether Trx1 or Trx2 was the electron donor. Thus, the kinetic properties of neither type I nor type II TPx differed between reactions mediated by Trx1 or Trx2.

The initial rate of NADPH oxidation was also measured at various concentrations of H_2O_2, cumene hydroperoxide, and *tert*-butyl hydroperoxide (not shown). The K_m values for H_2O_2 differed markedly between the reactions catalyzed by type I and II TPxs: 3 μM for the type I TPx reaction and 150 μM for the type II TPx reaction. The K_m values for cumene hydroperoxide (4 and 8 mM, respectively) and *tert*-butyl hydroperoxide (10 and 45 mM, respectively) were also lower for the type I TPx reaction than for the type II TPx reaction, although less markedly so than for H_2O_2. The V_{max} measured with each of the three peroxides was higher for type II TPx than type I TPx: 20 and 4.8 μmol min^{-1} mg^{-1} for the reduction of H_2O_2, 14 and 2.2 μmol min^{-1} mg^{-1} for the reduction of cumene hydroperoxide, and 17 and 2.4 μmol min^{-1} mg^{-1} for the reduction of *tert*-butyl hydroperoxide by type II and I TPx, respectively. The kinetic parameters are summarized in Table 1.

Role of Cysteine Residues. Previously, Cys^{47} and Cys^{170} of type I TPx were shown to form intermolecular disulfide linkages between two type I TPx monomers (*7*). Type II TPx contains three cysteine residues at amino acid positions 31, 62, and 120. To identify which of these three cysteines might form disulfide bonds, we individually replaced each with serine and expressed the corresponding recombinant type II TPx proteins (C31S, C62S, and C120S) as well as the recombinant wild-type protein (RWT) in *E. coli*. Crude bacterial extracts were subjected to SDS−PAGE in the absence or presence of 2-mercaptoethanol followed by immunoblot analysis with antibodies to type II TPx. A crude extract of *S. cerevisiae* was also analyzed as a source of yeast type II TPx (WT). Under reducing conditions, WT, RWT, C31S, C62S, and C120S were all detected at molecular sizes corresponding to the monomeric form (Figure 5A). However, under nonreducing conditions, the mobility patterns were complex. The major band of WT, RWT, and C31S proteins corresponded to the molecular size of a dimer, whereas the major band of C62S and C120S corresponded to the molecular size of the monomer (Figure 5B). Similar patterns of distribution between monomeric and dimeric forms were observed when purified proteins were visualized by Coomassie blue staining (not shown).

Type II TPx can also protect glutamine synthetase against damage by the thiol oxidation system (Fe^{3+}, O_2, and DTT), although its efficacy is less than that of type I TPx. The protection activities of WT, RWT, C31S, C62S, and C120S proteins were evaluated (Figure 6). WT, RWT, C31S, and C120S showed similar protection activities, whereas C62S failed to provide protection. However, measurement of peroxidase activity by monitoring the H_2O_2-dependent oxidation of NADPH revealed that both C62S and C120S were catalytically inactive, whereas WT, RWT, and C31S showed similar activities (Figure 7).

```
Type II                                          MSDLVNKK FPAGDYKFQY IAISQSDADS ESCKMPQTVE WSKLISENKK  48
                                                                                                    |
Type Ia                                                        MVAQ VQKQAPTFKK TAVVDGVFDE VSLDKYKGKY  34
Type Ib                                                        MVAE VQKQAPPFKK TAVVDGIFEE ISLEKYKGKY  34
1-Cys Prx  MFSRICSAQL KRTAWTLPKQ AHLQSQTIKT FATAPILCKQ FKQSDQPRLR INSDAPNFDA DTTVGKI... NFYDYLGDSW  77
                                                                  ** *      *

           VIITGAPAAF SPTCTVSHIP GYINYLDELV KEKEVDQVIV VTVDNPFANQ AWAKSLGVKD TTHIKFASDP GCAFTKSIGF 128
           |::    | || :  |    |
Type Ia    VVLAFIPLAF TFVCPTEIIA FSEAAKKFEE QGAQVLFAST DSEYSLLAWT NIPRKEGGLG PINIPLLADT NHSLSRDYGV 114
Type Ib    VVLAFVPLAF SFVCPTEIVA FSDAAKKFED QGAQVLFAST DSEYSLLAWT NLPRKDGGLG PVKVPLLADK NHSLSRDYGV 114
1-Cys Prx  GVLFSHPADF TPVCTTEVSA FAKLKPEFDK RNVKLIGLSV EDVESHEKWI QDIKEIAKVK NVGFPIIGDT FRNVAFLYD. 156
            **   *  *   ** **  * *      *         *      *   **                 *   *         *

Type II    ELAVGDG... ......VYWS GRWAMVVENG IVTYAAKETN PGTDVTVSSV ESVLAHL                          176

Type Ia    LIEEEGV... ......ALRG LFIIDPKGVI RHITINDLPV GRNVDEALRL VEAFQWTDKN GTVLPCNWTP GAATIKPTVE 185
Type Ib    LIEKEGI... ......ALRG LFIIDPKGII RHITINDLSV GRNVNEALRL VEGFQWTDKN GTVLPCNWTP GAATIKPDVK 185
1-Cys Prx  MVDAEGFKNI NDGSLKTVRS VFVIDPKKKI RLIFTYPSTV GRNTSEVLRV IDALQLTDKE GVVTPINWQP ADDVIIPPSV 236
               **             *   * ****  * * *      * *** *  **      * ***  * * * ** *     * *

Type Ia    DSKEYFEAAN K                                                                            196
Type Ib    DSKEYFKNAN N                                                                            196
1-Cys Prx  SNDEAKAKFG QFNEIKPYLR FTKSK                                                             261
             *
```

FIGURE 3: Comparison of the amino acid sequences of type II TPx and other previously known Prx proteins, type I TPx (type Ia), putative type I TPx (type Ib), and 1-Cys Prx (YBL0524). Identical and conserved residues between type I and type II TPxs are connected by vertical lines and colons, respectively. Because noticeable similarities between type I and II TPxs are found only in the short segments containing the amino-terminal essential cysteines, no vertical connections were made between randomly aligned residues. Amino acids that are conserved among type Ia, type Ib, and YBL0524 are marked by asterisks. The redox-sensitive cysteine residues of type II TPx (Cys^{62} and Cys^{120}) and type I TPx (Cys^{47} and Cys^{170}) are marked by bold letters. The regions of type II TPx corresponding to the sequenced tryptic peptides are overlined.

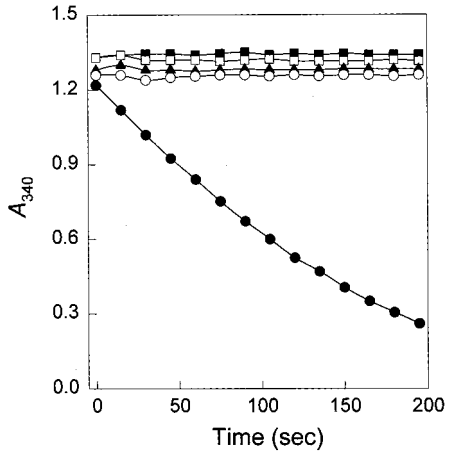

FIGURE 4: NADPH oxidation coupled by TR, Trx1, and type II TPx to the reduction of H_2O_2. NADPH oxidation was monitored as the decrease in A_{340} in a 0.5-mL reaction mixture containing 40 mM Hepes-NaOH (pH 7.0), 0.18 μM TR, 0.45 μM Trx1, 2.1 μM type II TPx, 0.25 mM NADPH, and 1 mM H_2O_2 (●). In other reaction mixtures, Trx1(□), type II TPx (○), TR (■), or H_2O_2 (▲) was omitted.

Effect of Type II TPx Overexpression on the Growth of E. coli Under Conditions of Oxidative Stress. The growth rates of *E. coli* expressing RWT and C62S forms of type II TPx were determined in the presence of *tert*-butyl hydroperoxide or cumene hydroperoxide. A filter paper containing the test oxidant was placed on a lawn of *E. coli* cells and growth inhibition was evaluated by the size of the clear zone surrounding the filter paper (Figure 8). Both *tert*-butyl hydroperoxide and cumene hydroperoxide inhibited the growth of control cells (transfected with vector only) to a greater extent than that of the cells expressing RWT, whereas the extent of growth inhibition was similar for the control and C62S-expressing cells.

DISCUSSION

The 195-amino acid type I TPx contains two cysteine residues, Cys^{47} and Cys^{170}, and shows sequence similarity to >40 proteins from a wide variety of species (*6*, *8*, *16*−*23*). These homologous proteins have been referred to as the peroxiredoxin (Prx) family (*6*); they were not termed the TPx family because not all use Trx as the hydrogen donor for reduction of peroxides (*24*).[2] One cysteine residue, which corresponds to Cys^{47} of yeast type I TPx, is conserved in all members of the Prx family. Although most family members contain a second cysteine that corresponds to Cys^{170} of yeast type I TPx, several lack such a residue; this criterion thus divides the Prx family into two subgroups (1-Cys and 2-Cys) (*6*). Nevertheless, the similarity between the proteins containing one or two conserved cysteine residues extends over the entire sequences. For example, a yeast open reading frame (YBL0524) encodes a 211-amino acid 1-Cys member that shows 31% sequence identity (51% similarity) to type I TPx over the entire length of the proteins (*6*).

The 176-amino acid type II TPx is a Trx-dependent peroxidase, but it shows no sequence similarity to type I TPx other than that in the short stretch of the sequences that precedes the conserved cysteine residues (Cys^{47} of type I

[2] K. Kim and S. G. Rhee, unpublished data.

Table 1: Kinetic Properties of Type I TPx and Type II TPx

	type I TPx			type II TPx		
substrates	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})	V_{max}/K_m	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})	V_{max}/K_m
Trx1	2	5.5	2.8	3	17	8.5
Trx2	3	5.5	1.8	2	16	8
H_2O_2	3	4.8	1.6	150	20	0.13
cumene hydroperoxide	4	2.2	0.55	8	14	1.8
tert-butyl hydroperoxide	10	2.4	0.24	45	17	0.38

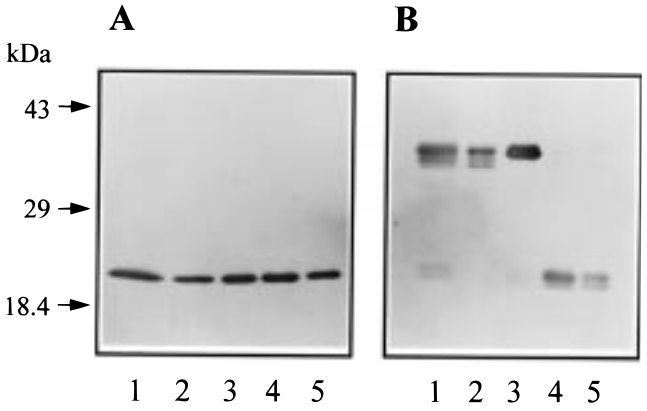

FIGURE 5: Immunoblot analysis of crude extracts of *S. cerevisiae* and of *E. coli* expressing wild-type and mutant recombinant forms of type II TPx. Crude extracts of *E. coli* (5 μg of protein in 10 μL) or *S. cerevisiae* (50 μg in 10 μL) were mixed with an equal volume of reducing sample buffer [0.125 M Tris-HCl (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, and 10% (v/v) 2-mercaptoethanol] (A) or nonreducing sample buffer (reducing sample buffer minus 2-mercaptoethanol) (B), heated at 95 °C for 3 min, and then subjected to SDS−PAGE on 12% gels. The separated proteins were transferred to nitrocellulose filters and subjected to immunoblot analysis with antibodies to type II TPx. Positions of molecular size standards are indicated in kilodaltons. Lane 1, *S. cerevisiae*; lanes 2−5, *E. coli* expressing recombinant wild-type (RWT), C31S, C62S, and C120S type II TPx proteins, respectively.

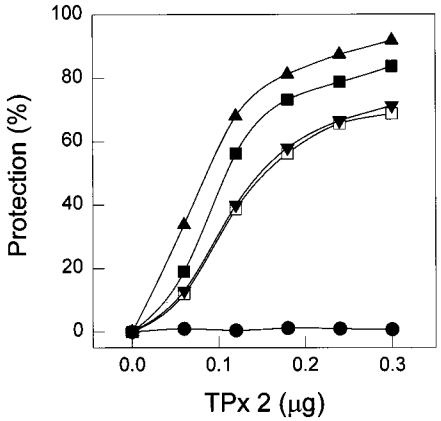

FIGURE 6: Effects of wild-type and mutant type II TPx proteins on inactivation of glutamine synthetase by the thiol oxidation system. Purified WT (□), RWT (▼), C31S (▲), C62S (●), and C120S (■) proteins were assayed for their ability to protect glutamine synthetase from inactivation as described in Experimental Procedures. Data are expressed as a percentage of the inactivation value in the absence of type II TPx.

TPx and Cys^{62} of type II TPx) (Figure 3). Similarly, type II TPx shows homology to YBL0524 only in short sequences that precede the conserved cysteines. Both type I TPx and type II TPx reduce various peroxides with hydrogen derived from Trx. In contrast, YBL0524, which is more similar to type I TPx than to type II TPx is, does not accept reducing

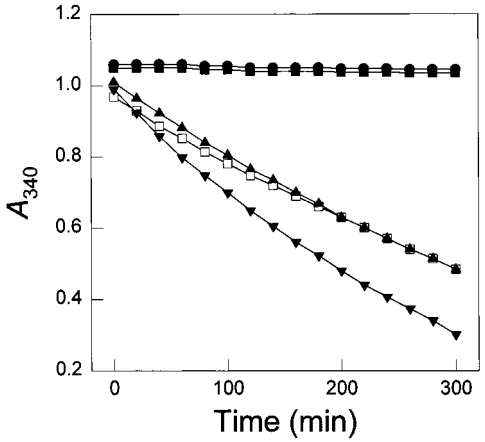

FIGURE 7: Peroxidase activity of wild-type and mutant type II TPx proteins. NADPH oxidation was monitored as the decrease in A_{340} in a 0.5-mL reaction mixture containing 50 mM Hepes-NaOH (pH 7.0), 0.18 μM TR, 8 μM Trx1, 0.2 mM NADPH, 1 mM H_2O_2, and 0.08 μM purified WT (□), RWT (▼), C31S (▲), C62S (●), and C120S (■) proteins.

equivalents from Trx; however, YBL0524 protects glutamine synthetase against damage by the thiol oxidation system and reduces H_2O_2 in the presence of DTT.[2]

Both yeast isoforms of Trx were indistinguishable in their hydrogen carrier function for both TPx enzymes. The K_m values for Trx1 and Trx2 are virtually identical between type I TPx and type II TPx, and the V_{max} values obtained by varying the concentration of Trx1 also are nearly identical with those obtained by varying the concentration of Trx2. The V_{max} for the reduction of H_2O_2 by type II TPx is approximately four times greater than observed with type I TPx, and the V_{max} values for cumene hydroperoxide and *tert*-butyl hydroperoxide are also higher for type II TPx than for type I TPx. However, all three peroxides show higher affinity for type I TPx than for type II TPx, the most pronounced (50-fold) difference being apparent with H_2O_2. Thus, the catalytic efficiency (V_{max}/K_m) of type II TPx toward H_2O_2 is approximately one-twelfth that of type I TPx. The lower catalytic efficiency of type II TPx is likely responsible for the fact that type II TPx was not readily detectable when column fractions were assayed on the basis of glutamine synthetase protection activity; most of the H_2O_2 generated as a result of the reduction of O_2 by DTT and Fe^{3+} in the assay solution would escape destruction by type II TPx and be further reduced to HO•. However, as expected, type II TPx also can protect glutamine synthetase when added at higher concentrations. In contrast to the glutamine synthetase assay, the peroxidase assay coupled to NADPH oxidation proved more sensitive for the detection of type II TPx because the assay mixture contains a saturating concentration (1 mM) of H_2O_2. The V_{max}/K_m ratio is greater for the reduction of H_2O_2 than for the reduction of alkyl hydroper-

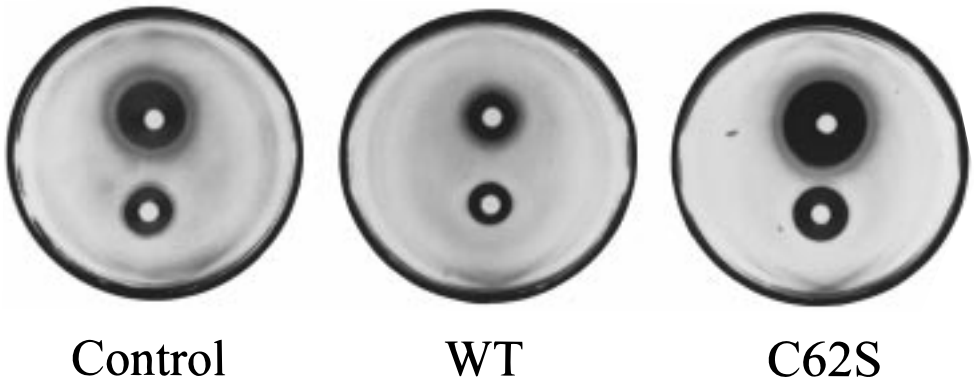

FIGURE 8: Effects of type II TPx overexpression in *E. coli* on the growth inhibition exerted by alkyl hydroperoxides. Disks containing 5 μL of either 100 mM *tert*-butyl hydroperoxide (upper disks) or 150 mM cumene hydroperoxide (lower disks) were placed on lawns of *E. coli* transfected with vector alone or of *E. coli* expressing RWT or C62S forms of type II TPx, as indicated. The plates were incubated overnight at 37 °C.

oxides in type I TPx, whereas it is smaller in type II TPx. Therefore, in yeast, lipid peroxides and H_2O_2 might be preferentially eliminated by type II TPx and type I TPx, respectively. In this connection, a protein showing 42% sequence identity to type II TPx was identified as a peroxisomal membrane protein in *Candida boidinii* (*25*), whereas type I TPx was found in cytosol (*2*).

We showed previously that the Cys^{47}—SH group of type I TPx is the site of substrate peroxide reduction and is oxidized directly by peroxide presumably to cysteine sulfenic acid (Cys^{47}—OH) (*10*). The Cys^{47}—OH then reacts with Cys^{170}—SH of another type I TPx molecule to produce H_2O and an intermolecular disulfide bond, which is subsequently reduced by Trx. Thus, both Cys^{47} and Cys^{170} are essential for Trx-dependent peroxidase activity and the oxidized type I TPx is a dimer linked by disulfide bonds between Cys^{47} and Cys^{170} (reduced type I TPx also exists as a dimer under nondenaturing conditions) (*7, 10*). In the glutamine synthetase protection assay mixture, DTT reduces the disulfide bond between Cys^{47} and Cys^{170}. In contrast to the Trx-dependent peroxidase reaction, the glutamine synthetase protection reaction requires Cys^{47} but not Cys^{170}, presumably because the sulfhydryl group of DTT can reduce Cys^{47}—OH to Cys^{47}—SH or replace Cys^{170} in the formation of a disulfide bond with Cys^{47}—OH (*7, 10*).

Type II TPx contains three cysteine residues (Cys^{31}, Cys^{62}, and Cys^{120}), of which Cys^{62} is essential for both Trx-dependent peroxidase and glutamine synthetase protection reactions, whereas Cys^{120} is required only for Trx-dependent peroxidase activity. Mutation of Cys^{31} affected neither of the two activities substantially. Furthermore, on oxidation, mutants lacking either Cys^{62} or Cys^{120} failed to form a dimer linked by disulfide bonds, whereas WT type II TPx and the mutant lacking Cys^{31} did form dimers. These results suggest that the mechanism by which type II TPx reduces peroxide is similar to that of type I TPx: Cys^{62}—SH is oxidized by peroxide to Cys^{62}—OH, which subsequently reacts with Cys^{120}—SH of another type II TPx molecule to form an intermolecular disulfide bond. In the absence of Cys^{120}—SH, a thiol such as DTT can support peroxidase function by reducing the Cys^{62}—OH or by forming a disulfide bond with Cys^{62}—OH. This similarity in mechanism may be relevant to the fact that the amino acid sequences preceding Cys^{62} of type II TPx and Cys^{40} of type I TPx are partly conserved, whereas the sequence surrounding Cys^{120} of type II TPx shows no homology to that surrounding Cys^{170} of type I TPx.

The proposed reaction intermediate with an intermolecular disulfide linkage(s) of Cys^{62} and Cys^{120} is also consistent with the results of the SDS−PAGE analysis of WT and mutant type II TPx proteins. In the reducing gels visualized by immunoblot analysis and Coomassie blue staining, the wild type and three mutants (C31S, C62S, and C120S) were all detected as monomers. However, in nonreducing gels, wild type and C31S were detected mainly in the dimeric region, whereas C62S and C120S were in the monomeric region. These results indicate that oxidation of type II TPx results in a dimer formed through a disulfide linkage of Cys^{62} and Cys^{120}. In nonreducing gels, especially in those visualized by immunoblot, wild type and C31S yielded several minor bands in the dimeric region. These are likely due to dimers with only one disulfide linkage of Cys^{62} and Cys^{120} and with disulfide linkages other than Cys^{62}—S−S−Cys^{120} such as Cys^{62}—S−S−Cys^{62} or Cys^{62}—S−S−Cys^{31}. The multiple monomeric bands seen in the nonreducing gels of C62S and C120S might be attributable to the fact that their cysteines might undergo oxidation to Cys—SOH, which may be further oxidized to Cys—SOOH or may form on intramolecular disulfide linkage with the remaining Cys—SH.

With the identification of type II TPx, four distinct Prx proteins, two type I TPx, one type II TPx, and 1-Cys Prx (YBL0524), have now been shown to be expressed in yeast. Mammalian cells express four different Prx proteins that exhibit a sequence identity of 60 to 70% with type I TPx (*22, 23*). All four of these proteins are products of separate genes, contain two conserved Cys residues that correspond to Cys^{47} and Cys^{170} of type I TPx, and reduce peroxides with the use of electrons from Trx (*22, 23*). A mammalian homologue of 1-Cys Prx has also been characterized recently (*24*): Upon oxidation, the conserved cysteine is converted to and remains as Cys-SOH until reduced by DTT or an unidentified physiological electron donor. Given these findings, it will be of interest to see whether type II TPx homologues are also present in mammalian tissues.

Finally, a peroxidase role for type II TPx in vivo is indicated by the observation that the growth of *E. coli* cells overexpressing WT type II TPx was inhibited to a lesser extent in the presence of *tert*-butyl hydroperoxide or cumene hydroperoxide than that of control cells, whereas the extent

of inhibition was similar for control cells and for cells overexpressing the C62S mutant of type II TPx.

REFERENCES

1. Halliwell, B., and Gutterridge, J. M. C. (1989) *Free Radicals in Biology and Medicine*, 2nd ed., pp 86−179, Clarendon Press, Oxford, U.K.
2. Kim, K., Rhee, S. G., and Stadtman, E. R. (1985) *J. Biol. Chem. 260*, 15394−15397.
3. Kim, K., Kim, I.-H., Lee, K. Y., Rhee, S. G., and Stadtman, E. R. (1988) *J. Biol. Chem. 263*, 4704−4711.
4. Yim, M. B., Chae, H. Z., Rhee, S. G., Chock, P. B., and Stadtman, E. R. (1994) *J. Biol. Chem. 269*, 1621−1626.
5. Chae, H. Z., Kim, I.-H., Kim, K., and Rhee, S. G. (1993) *J. Biol. Chem. 268*, 16815−16821.
6. Chae, H. Z., Robison, K., Poole, L. B., Church, G., Strotz, G., and Rhee, S. G. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 7017−7021.
7. Chae, H. Z., Uhm, T. B., and Rhee, S. G. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 7022−7026.
8. Rhee, S. G., and Chae, H. Z. (1994) *Mol. Cells 4*, 137−142.
9. Netto, L. E. S., Chae, H. Z., Kang, S.-W., Rhee, S. G., and Stadtman, E. R. (1996) *J. Biol. Chem. 271*, 15315−15321.
10. Chae, H. Z., Chung, S. J., and Rhee, S. G. (1994) *J. Biol. Chem. 269*, 27670−27678.
11. Kwon, S. J., Park, J.-W., Choi, W.-K., Kim, I. H., and Kim, K. (1994) *Biochem. Biophys. Res. Commun. 201*, 8−15.
12. Rhee, S. G., Chock, P. B., and Stadtman, E. R. (1985) *Methods Enzymol. 113*, 213−241.
13. Gan, Z.-R. (1991) *J. Biol. Chem. 266*, 1692−1696.
14. Muller, E. G. D. (1992) *Yeast 8*, 117−120.
15. Thurman, R. G., Ley, H. G., and Scholz, R. (1972) *Eur. J. Biochem. 25*, 420−430.
16. Lim, Y. S., Cha, M. K., Kim, H. K., and Kim, I. H. (1994) *Gene 140*, 279−284.
17. Prospéri, M.-T., Fervus, D., Karczinski, I., and Goubin, G. (1993) *J. Biol. Chem. 268*, 11050−11056.
18. Watabe, S., Kohno, H., Kouyama, H., Hiroi, T., Yago, N., and Nakazawa, T. (1994) *J. Biochem. 115*, 648−654.
19. Iwahara, S.-I., Satoh, H., Song, D.-X., Webb, J., Burlingame, A. L., Nagae, Y., and Muller-Eberhard, U. (1995) *Biochemistry 34*, 13398−13406.
20. Yamamoto, T., Matsui, Y., Natori, S., and Obinata, M. (1989)- *Gene 80*, 337−343.
21. Ishii, T., Yamada, M., Sato, H., Matsue, M., Taketani, S., Nakayama, K., Sugita Y., and Bannai, S. (1993) *J. Biol. Chem. 268*, 18633−18636.
22. Jin, D.-Y., Chae, H. Z., Rhee, S. G., and Jaeng, K.-T. (1997) *J. Biol. Chem. 272*, 30952−30961.
23. Kang, S. W., Chae, H. Z., Seo, M. S., Kim, K., Baines, I. C., and Rhee, S. G. (1998) *J. Biol. Chem. 273*, 6297−6302.
24. Kang, S. W., Baines, I. C., and Rhee, S. G. (1998) *J. Biol. Chem. 273*, 6303−6311.
25. Garrels, J. I., Futcher, B., Kobayashi, R., Latter, G. I., Schwender, B., Volpe, T., Warner, J. R., and McLaughlin, C. S. (1989) *J. Biol. Chem. 264*, 13929−13937.

BI9817818