Characterization of a Mammalian Peroxiredoxin That Contains One Conserved Cysteine*

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A new type of peroxidase enzyme, named thioredoxin peroxidase (TPx), that reduces \( \text{H}_2\text{O}_2 \) with the use of electrons from thioredoxin and contains two essential cysteines was recently identified. TPx homologs, termed peroxiredoxin (Prx), have also been identified and include several proteins, designated 1-Cys Prx, that contain only one conserved cysteine. Recombinant human 1-Cys Prx expressed in and purified from \textit{Escherichia coli} has now been shown to reduce \( \text{H}_2\text{O}_2 \) with electrons provided by dithiothreitol. Furthermore, human 1-Cys Prx transiently expressed in NIH 3T3 cells was able to remove intracellular \( \text{H}_2\text{O}_2 \) generated in response either to the addition of exogenous \( \text{H}_2\text{O}_2 \) or to treatment with platelet-derived growth factor. The conserved Cys\(^{47}-\text{SH} \) group was shown to be the site of oxidation by \( \text{H}_2\text{O}_2 \). Thus, mutation of Cys\(^{47} \) to serine abolished peroxidase activity. Moreover, the oxidized intermediate appears to be Cys–SOH. In contrast to TPx, in which one of the two conserved cysteines is oxidized to Cys–SOH and then immediately reacts with the second conserved cysteine of the second subunit of the enzyme homodimer to form an intermolecular disulfide, the Cys–SOH of 1-Cys Prx does not form a disulfide. Neither thioredoxin, which reduces the disulfide of TPx, nor glutathione, which reduces the Cys–SeOH of oxidized glutathione peroxidase, was able to reduce the Cys–SOH of 1-Cys Prx and consequently could not support peroxidase activity. Human 1-Cys Prx was previously shown to exhibit a low level of phospholipase A\(_2 \) activity at an acidic pH; the enzyme was thus proposed to be lysosomal, and Ser\(^{32} \) was proposed to be critical for lipase function. However, the mutation of Ser\(^{32} \) or Cys\(^{47} \) has now been shown to have no effect on the lipase activity of 1-Cys Prx, which was also shown to be a cytosolic protein. Thus, the primary cellular function of 1-Cys Prx appears to be to reduce peroxides with the use of electrons provided by an as yet unidentified source; the enzyme therefore represents a new type of peroxidase.

Members of the family of peroxiredoxin (Prx)\(^1 \) proteins show amino acid sequence homology to thioredoxin peroxidase (TPx), a 25-kDa peroxidase, initially identified in yeast, that reduces \( \text{H}_2\text{O}_2 \) with the use of electrons provided by thioredoxin (Trx) (1–4). More than 40 members of the Prx family have been identified in a wide variety of organisms ranging from prokaryotes to mammals (1), although it is not known whether all of these proteins actually catalyze the reduction of peroxides.

Yeast TPx exists as a homodimer and contains two essential Cys residues, Cys\(^{47} \) and Cys\(^{170} \), in each subunit. The Cys\(^{47}-\text{SH} \) group is the primary site of oxidation by \( \text{H}_2\text{O}_2 \), and the oxidized Cys\(^{47} \) (probably Cys–SOH) rapidly reacts with Cys\(^{170}-\text{SH} \) of the other subunit to form an intermolecular disulfide. This disulfide is subsequently reduced by Trx. Mutant TPx proteins that lack either Cys\(^{47} \) or Cys\(^{170} \) therefore do not exhibit Trx-coupled peroxidase activity (2, 5). Another well characterized member of the Prx family is alkyl hydroperoxide reductase from \textit{Salmonella typhimurium} (6). This enzyme also contains two conserved cysteines that correspond to Cys\(^{47} \) and Cys\(^{170} \) of yeast TPx, and it reduces alkyl hydroperoxides with the use of electrons donated by the 57-kDa flavoprotein alkyl hydroperoxide reductase C (1, 7, 8).

Although most Prx family members contain two conserved cysteines that correspond to Cys\(^{47} \) and Cys\(^{170} \) of yeast TPx, seven Prx proteins from various organisms contain only one conserved cysteine residue, corresponding to Cys\(^{47} \) of yeast TPx (Fig. 1). Thus, members of the Prx family can be divided into two subgroups, 1-Cys and 2-Cys, the latter of which includes TPx and alkyl hydroperoxide reductase C. The full-length cDNA for a human 1-Cys Prx (clone HA0683) was identified as the result of a sequencing project with human myeloid cell cDNA (9). In addition to this 1-Cys Prx, human cells express three distinct 2-Cys Prx proteins, which have been referred to as TPx I to III because they reduce \( \text{H}_2\text{O}_2 \) in the presence of Trx (10). Whether 1-Cys Prx proteins also catalyze the reduction of peroxides and, if so, the identity of the electron donor have remained unknown.

Recently, the human 1-Cys Prx protein was shown to be a \( \text{Ca}^{2+} \)-independent phospholipase A\(_2 \) (PLA\(_2 \)) that exhibits maximal activity at pH 4 (11). This protein contains a five-amino acid motif, Gly-X-Ser-X-Gly (where X is any amino acid), that is present in many neutral lipases (12). We have now shown that recombinant human 1-Cys Prx mediates the reduction of \( \text{H}_2\text{O}_2 \) with the use of electrons from a nonphysiological donor, dithiothreitol (DTT). Although the physiological electron donor remains unknown, overexpression of the protein in NIH 3T3 cells

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§ The abbreviations used are: Prx, peroxiredoxin (the Prx family was previously referred to as the alkyl hydroperoxide reductase C-thiol-specific antioxidant family); TPx, thioredoxin peroxidase (previously referred to as thiol-specific antioxidant or TSA); Trx, thioredoxin; PLA\(_2 \), phospholipase A\(_2 \); DTT, dithiothreitol; GPx, glutathione peroxidase; GS, glutamine synthetase; DMEM, Dulbecco's modified Eagle's medium; MEM, minimum essential medium; DCFH-DA, 2,7'-dichlorofluorescein diacetate; DCF, 2,7'-dichlorofluorescein; PDGF, platelet-derived growth factor; PDGF-AB, PDGF AB heterodimer; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); TNB, 5-thio-2-nitrobenzoic acid; WT, wild type.
Peroxiredoxin with One Conserved Cysteine

EXPERIMENTAL PROCEDURES

Materials—Glutamine synthetase (GS) was purified from Escherichia coli as described (15). Recombinant human Trx, rat liver Trx and NADH peroxidase, all of which contain a cysteine or selenocysteine as the primary site of reaction with peroxides.

Bacterial Expression of Human 1-Cys Prx—A full-length 1-Cys Prx of the human protein are indicated. The second amplification reaction was performed with more internal forward primer (5' CAG CCAG CAC CAT CAC-3').

Molecular Probes. A stock solution of FeCl₃ was prepared in 0.1 M HCl, and 2-mercaptoethanol, and Lipofectamine were obtained from Life Technologies, Inc., and 2-mercaptoethanol, and Lipofectamine were obtained from Life Technologies, Inc.

Essential medium (MEM), Opti-MEM, fetal bovine serum, calf serum, 1-Cys Prx. Dulbecco’s modified Eagle’s medium (DMEM), minimum essential medium (MEM), Opti-MEM, fetal bovine serum, calf serum, 1-Cys Prx.

FIG. 1. Sequence alignment of 1-Cys Prx family members. The deduced amino acid sequences of Homo sapiens (human) (GenBankTM accession number D14662), Mus musculus (mouse) (accession number Y12883), Onchocerca volvulus (nematode) (accession number U31052), Hordeum vulgare (barley) (accession number X76605), Orzya sativa (rice) (accession number D63917), Tortula ruralis (moss) (accession number U40818), Saccharomyces cerevisiae (yeast) (accession number Z23261), and Sulfolobus sp. (archaea) (accession number U56479) were aligned with the use of the Genetics Computer Group’s PILEUP program. The conserved cysteine (●) were indicated, and residue numbers are indicated on the right.

revealed a peroxidase function in vivo. We also compared the catalytic mechanism of 1-Cys Prx with those of TPx, glutathione peroxidase (GPx), and NADH peroxidase, all of which contain a cysteine or selenocysteine as the primary site of reaction with peroxides.

Purification of Recombinant 1-Cys Prx Proteins—Escherichia coli strain BL21(DE3) harboring the appropriate plasmid was cultured at 37 °C overnight in 100 ml of LB medium supplemented with ampicillin (100 μg/ml) and then transferred to 10 liters of fresh LB medium in a Microferm fermentor (New Brunswick Scientific). When the optical density of the culture at 600 nm reached 0.6–0.8, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.4 mM. After incubation for 3 h, the cells were collected by centrifugation, frozen in liquid nitrogen, and stored at −70 °C until use. The 1-Cys Prx proteins were present in the soluble fraction of the bacterial cells (data not shown). During purification, recombinant 1-Cys Prx proteins were detected by immunoblot analysis with specific polyclonal antibodies.

Frozen cells (4 g) were suspended in 20 ml of buffer A (20 mM imidazole, 500 mM NaCl, 5 mM 2-mercaptoethanol, 5 mM MgCl₂, pH 8.0) and disrupted by sonication. The resulting cell extract was centrifuged at 12,000 × g for 30 min. Streptomycin sulfate was added to the supernatant to a final concentration of 1%, and, after 30 min at 4 °C, the mixture was centrifuged at 12,000 × g for 30 min. Solid ammonium sulfate was slowly added, at 4 °C with stirring, to the resulting supernatant to 80% saturation, after which the mixture was stirred for 1 h. The resulting precipitate was collected by centrifugation at 15,000 × g for 30 min and dissolved in 10 ml of buffer A containing 0.5 M (NH₄)₂SO₄. Insoluble material was removed by centrifugation at 15,000 × g for 30 min, and the resulting supernatant was fractionated by high performance liquid chromatography on a TSK phenyl 5PW column (21.5 by 150 mm) that had been equilibrated with buffer A containing 1 M (NH₄)₂SO₄. Proteins were eluted with a descending gradient of ammonium sulfate from 1 to 0 M over 60 min at a flow rate of 5 ml/min. Fractions of 1 ml were collected, and those (fractions 47–51) corresponding to the peak of 1-Cys Prx were pooled, dialyzed against 2 liters of distilled water, and concentrated in an Amicon concentrator. The concentrated sample was applied to a Mono Q HR10/10 column (Pharmacia Biotech Inc.) that had been equilibrated with buffer B, and the column was washed with the

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same buffer for 10 min. 1-Cys Prx was detected in the flow-through buffer, and those fractions containing the protein were pooled, dialyzed against 2 liters of buffer A, and stored at −70 °C until use. The mutant C47S, C91S, S32A, and S32G proteins were prepared by a procedure similar to that for the wild-type enzyme. If necessary, DTT and EDTA were removed from the protein preparation before use by dialysis or by desalting on a PD-10 column.

**GS Protection Assay**—GS protection by 1-Cys Prx was measured as described previously (17) with a slight modification. The 25-μl reaction mixture, containing 0.5 μg of GS, 10 mM DTT or ascorbate, 5 μM FeCl₃, 50 mM Hepes-NaOH (pH 7.0), and various concentrations of 1-Cys Prx, was incubated at 37 °C for 10 min, after which 1 ml of γ-glutamyltransferase assay mixture was added, and the remaining activity of GS was measured at 37 °C for 3 min.

**Cell Culture and Transfection**—Mouse NIH 3T3 fibroblasts and human A431 epidermoid carcinoma cells were cultured in DMEM containing penicillin (100 units/ml), streptomycin (100 units/ml), amphotericin B (0.25 μg/ml), and either 10% calf serum or 10% fetal bovine serum, respectively. Cells were continuously passaged for 3 months after thawing. For transfection, cells were plated at a density of 3 × 10⁵ cells/ml, allowed to recover for 24 h, and then incubated with 4 μg of appropriate DNA and 20 μl of Lipofectamine in 3 ml of Opti-MEM. After 6 h, 3 ml of DMEM containing 20% calf serum were added to the transfection mixture, and the cells were incubated for an additional 18 h. The medium was then aspirated, and cells were incubated for 18 h in fresh DMEM containing 0.5% calf serum before measurement of H₂O₂.

**Assay of Intracellular Hydrogen Peroxide**—Intracellular H₂O₂ generation was measured with the fluorescent dye DCFH-DA as described (18), with a slight modification. Briefly, serum-deprived transfected cells were washed with MEM without phenol red and treated for 5 min with H₂O₂ (10 μM) or for 10 min with PDGF-AB (5 ng/ml) in the same medium. The cells were then once with Krebs-Ringer solution and then incubated in the same solution, to which DCFH-DA (5 μg/ml) was added immediately before use. Culture dishes were sealed with paraffin film and placed in a CO₂ incubator at 37 °C for 5 min, after which DCF fluorescence was measured with a Zeiss Axiovert 135 inverted microscope equipped with a X20 Neofluor objective and Zeiss LSM410 confocal attachment. Photo-oxidation of DCFH was avoided by collecting the fluorescence image by the use of a single rapid scan (1-s scan; four-line average; total scan time of 4.3 s) with identical parameters, such as contrast and brightness, for all samples. The cells were then imaged by differential interference contrast microscopy. Five groups of 10–20 cells were randomly selected from the image of each sample, and the profiles of the selected cells were individually traced in the differential interference image. The mean fluorescence intensity of each profile was then measured by overlaying the fluorescence image.

**Assay of PLA₂ Activity**—NIH 3T3 cells transfected with the appropriate DNA as described above were incubated for 24 h in DMEM supplemented with 10% calf serum. Cells were washed with and scraped into ice-cold phosphate-buffered saline and collected by centrifugation at 500 × g for 5 min. The resulting cell pellet was suspended in 0.5 ml of extraction buffer (20 mM Hepes-NaOH (pH 7.0), 1 mM EDTA, 5 mM DTT, and 10% (v/v) glycerol) and sonicated. The cell lysate was centrifuged at 15,000 × g for 10 min, and the resulting supernatant was subjected to a batch-type purification by mixing with 200 μl of DEAE-Sepharose (Pharmacia) that had been equilibrated with extraction buffer. The unbound fraction was collected after brief centrifugation, and PLA₂ activity in this fraction was measured with mixed micelles that were prepared by sonication of a mixture of 1 ml of 1-palmitoyl-2-[9,10-³H]palmitoyl-sn-glycerol-3-phosphocholine and 4 ml Triton X-100 in saline. The reaction mixture contained 50 mM sodium acetate (pH 4.0), 1 mM EDTA, 0.1 mM [³H]phosphatidic acid (0.5 μCi), and PLA₂ source in a final volume of 250 μl. After incubation for 1 h at 37 °C, the lipid products were analyzed by thin layer chromatography for [³H]palmitic acid as described by Kim et al. (11).

**RESULTS**

**Chemical Properties of Cysteine Residues of 1-Cys Prx**—The peroxidase reaction of TPx proteins requires both conserved cysteine residues, because the oxidized enzyme intermediate generated during the catalytic cycle is a dimer in which the subunits are linked by one or two intermolecular disulfide bonds between Cys⁴⁷ and Cys⁷⁰ (see Fig. 10B). Human 1-Cys Prx contains one cysteine, at amino acid position 91, in addition to the conserved Cys⁴⁷. To investigate whether 1-Cys Prx is indeed a peroxidase and, if so, whether both Cys⁴⁷ and Cys⁹¹ are required for activity, we individually replaced each cysteine residue with serine. The corresponding recombinant mutant (C47S and C91S) and wild-type (WT) proteins were expressed in E. coli and purified from the soluble fraction of the bacterial cells. The purified proteins were heated at 95 °C for 5 min in SDS sample buffer, in the absence or presence of DTT, and analyzed by SDS-PAGE. In the absence of DTT, cysteine residues would be expected to be oxidized during heating. However, the WT, C47S, and C91S proteins were all detected at molecular sizes corresponding to the monomeric form regardless of the presence or absence of DTT (Fig. 2A). This result suggests that, unlike TPx enzymes, 1-Cys Prx does not form intermolecular disulfide linkages upon oxidation. Whereas reduced (DTT-treated) forms of the WT, C47S, and C91S proteins showed identical electrophoretic mobilities, oxidized WT migrated slightly faster than the oxidized mutant proteins. Treatment of the WT enzyme with increasing concentrations of DTT resulted in a gradual shift in the protein band from the position of higher mobility to that of lower mobility (Fig. 2B). These observations suggest that oxidation of WT might result in the formation of an intramolecular linkage between Cys⁴⁷ and Cys⁹¹ and thereby increase the compactness of the protein conformation.

In the absence of DTT, cysteine residues of 1-Cys Prx are likely to be oxidized to cysteine sulfenic acid (Cys-SOH). Sulfenic acid readily undergoes condensation with a thiol to form a disulfide (19). However, a sulfenic acid group of 1-Cys Prx might be able to react with the other cysteine only in the denatured conformation of the protein; spatial separation of the reactive groups in the native conformation may prevent their condensation. To investigate this possibility, we induced oxidation of 1-Cys Prx by exposure to H₂O₂ and then incubated the protein in the absence or presence of Ellman’s reagent (5,5’-dithiobis(2-nitrobenzoic acid) (DTNB)) before oxidation by heat followed by nonreducing SDS-PAGE analysis. Most of the protein that had not been labeled with DTNB migrated in the higher mobility position, whereas most of that labeled by DTNB migrated in the lower mobility position (Fig. 3A). In a related experiment, the oxidized, labeled 1-Cys Prx as well as...
the oxidized, unlabeled 1-Cys Prx were denatured and digested with trypsin. The resulting peptides were separated on a high pressure liquid chromatography C18 column (Fig. 3B). Comparison of the elution profiles on the top and bottom of Fig. 3B indicate that peptide I, which is present mainly in the tryptic digests of the unlabeled 1-Cys Prx, is a candidate for a peptide containing a disulfide. Indeed, after reduction by DTT, peptide I yielded two new peptides, I-1 and I-2, which contained Cys47 containing a disulfide. Indeed, after reduction by DTT, peptide I, which is present mainly in the tryptic digest of the 1-Cys Prx, is a candidate for a peptide containing a disulfide. This latter explanation, given that it remained unmodified even in the absence of H2O2, suggests that the H2O2-oxidized cysteine is distinct from the DTNB-reactive residue. Moreover, the H2O2-sensitive cysteine appears physically inaccessible to DTNB in the native conformation, given that it remained unmodified even in the absence of H2O2. With denatured protein that had not been exposed to H2O2, 1.8 molecules of TNB were generated per 1-Cys Prx molecule, suggesting that both Cys47 and Cys91 are available for modification. Treatment of 10 μM 1-Cys Prx with 5 or 7.5 μM H2O2 reduced the number of TNB molecules per denatured 1-Cys Prx molecule to 0.75 and 0.25, respectively, indicating that sulfenic acid resulting from the oxidation of one Cys–SH reacted with the other Cys–SH to form a disulfide in the denatured protein. At higher H2O2 concentrations, the number of TNB residues released per denatured 1-Cys Prx molecule increased, probably because the sulfenic acid was further oxidized to sulfonic acid (Cys–SO3H) by excess H2O2 and consequently could not form a disulfide with the DTNB-reactive cysteine. This latter explanation was supported by nonreducing SDS-PAGE analysis of the H2O2-treated protein (Fig. 4B); the intensity of the higher measured the number of cysteine residues that could be modified by DTNB before and after denaturation with guanidine hydrochloride (Fig. 4A). Approximately one 5-thio-2-nitrobenzoic acid (TNB) residue was detected per native protein molecule at concentrations of H2O2 from 0 to 100 μM, suggesting that the H2O2-oxidized cysteine is distinct from the DTNB-reactive residue. Moreover, the H2O2-sensitive cysteine appears physically inaccessible to DTNB in the native conformation, given that it remained unmodified even in the absence of H2O2. With denatured protein that had not been exposed to H2O2, 1.8 molecules of TNB were generated per 1-Cys Prx molecule, suggesting that both Cys47 and Cys91 are available for modification. Treatment of 10 μM 1-Cys Prx with 5 or 7.5 μM H2O2 reduced the number of TNB molecules per denatured 1-Cys Prx molecule to 0.75 and 0.25, respectively, indicating that sulfenic acid resulting from the oxidation of one Cys–SH reacted with the other Cys–SH to form a disulfide in the denatured protein. At higher H2O2 concentrations, the number of TNB residues released per denatured 1-Cys Prx molecule increased, probably because the sulfenic acid was further oxidized to sulfonic acid (Cys–SO3H) by excess H2O2 and consequently could not form a disulfide with the DTNB-reactive cysteine. This latter explanation was supported by nonreducing SDS-PAGE analysis of the H2O2-treated protein (Fig. 4B); the intensity of the higher
mobility (intramolecular disulfide-containing) band peaked at 7.5 μM H₂O₂, gradually decreasing as the concentration of H₂O₂ increased further.

To identify the DTNB-reactive residue, we compared measurements of TNB release for WT, C47S, and C91S proteins that had not been exposed to H₂O₂. The numbers of TNB residues released per molecule of native or denatured 1-Cys Prx were 1.1 and 1.8 for WT, 0.8 and 0.8 for C47S, and 0.1 and 1.1 for C91S, respectively. These results suggest that Cys⁹¹–SH is the site of DTNB modification in the native enzyme and that Cys⁴⁷–SH becomes available only after denaturation.

In Vitro Peroxidase Activity of 1-Cys Prx—In the presence of an electron donor such as DTT or ascorbate, Fe³⁺ catalyzes the reduction of O₂ to H₂O₂, which is further converted to hydroxyl radicals (HO⁺) by the Fenton reaction (20). Both the DTT oxidation system (DTT, Fe³⁺, and O₂) and ascorbate oxidation system (ascorbate, Fe³⁺, and O₂) therefore inflict damage on various enzymes, including GS, and this damage can be prevented by an enzyme that eliminates H₂O₂. Yeast and mammalian TPx enzymes protect GS from damage by the DTT oxidation system but not by the ascorbate system; the intramolecular disulfide of oxidized TPx can be reduced by DTT but not by ascorbate (2). We therefore investigated whether 1-Cys Prx can protect GS from damage induced by these metal-catalyzed oxidation systems (Fig. 5A). Similar to TPx, 1-Cys Prx protected GS from the DTT system but not from the ascorbate system. For a reason that is not presently clear, C91S was slightly more effective than WT in protecting GS from the DTT system; C47S did not provide any such protection. These results suggest that 1-Cys Prx is indeed a peroxidase, that the peroxidase reaction involves the oxidation of Cys⁴⁷ but not Cys⁹¹, and that Cys⁴⁷–SOH can be converted back to Cys–SH by DTT but not by ascorbate.

We compared the peroxidase activities toward H₂O₂ of 1-Cys Prx proteins (WT, C47S, and C91S) and TPx by directly monitoring the decrease in H₂O₂ concentration in the presence of DTT (Fig. 5B). At a concentration of 25 μM, the rate of H₂O₂ removal by 2 mM DTT alone was negligible. The addition of WT, C91S, or TPx markedly increased the rate of H₂O₂ removal, whereas C47S had no effect. The initial rate of the reaction in the presence of 1.9 μM WT (or C91S) was faster than that in the presence of 4.5 μM TPx. However, the rate of H₂O₂ reduction by WT (or C91S) decreased gradually with time, whereas the rate of the TPx-mediated reaction remained virtually constant. When the concentration of H₂O₂ was increased to 100 μM, the reaction rates for WT and C91S decreased rapidly, reaching after 2 min a value similar to that for DTT alone (Fig. 5C). This observation is consistent with the notion that the sulfenic acid intermediate of 1-Cys Prx is readily oxidized by H₂O₂ to sulfonic acid, which cannot be reduced by DTT.

The physiological electron donor for the catalytic function of TPx has been shown to be Trx. Both the GS protection activity and peroxidase activity (toward H₂O₂) of TPx enzymes are markedly higher in the presence of the Trx system (Trx, Trx reductase, and NADPH) than in the presence of the nonphysiological electron donor DTT (2). GSH does not support the catalytic activity of TPx (17). We therefore examined 1-Cys Prx for GS protection activity in the presence of the Trx system or GSH. Taking advantage of the fact that the ascorbate oxidation system can inactivate GS but cannot provide electrons required for 1-Cys Prx function, we measured GS activity after incubation with a mixture of the ascorbate oxidation system with either the Trx system or the GSH system (GSH, glutathione reductase, and NADPH) (Fig. 6A). The activity of 1-Cys Prx was compared in these experiments with those of TPx and GPx. As expected, neither 1-Cys Prx, TPx, nor GPx protected GS from damage induced by the ascorbate system (data not shown). However, all three peroxidases protected against damage by the DTT system. It was previously shown that DTT can...
**In Vivo Peroxidase Activity of 1-Cys Prx**—To investigate the potential role of cysteine residues of 1-Cys Prx in PLA₂ activity, we attempted to measure this enzyme activity with the recombinant WT, C47S, and C91S proteins purified from *E. coli*. However, we failed to detect any substantial PLA₂ activity associated with the recombinant proteins. We then transiently expressed the three proteins in NIH 3T3 cells. Because Ser³² was previously proposed to constitute the PLA₂ active site (11), we also separately expressed two 1-Cys Prx mutants in which Ser 32 was replaced by alanine (S32A) or glycine (S32G). Because human 1-Cys Prx does not bind to DEAE-Sephacel, the WT protein was omitted when DTT was added as electron donor, but not by 1-Cys Prx. These results suggest that neither Trx nor GSH can efficiently replace GSH for the reduction of oxidized GPx (21). When the Trx system was added to the ascorbate system, TPx provided protection, but 1-Cys Prx did not. With the addition of exogenous H₂O₂ resulted in an increase in DCF fluorescence in NIH 3T3 cells transfected with vector alone (Fig. 7B). However, overexpression of WT, but not of C47S, prevented the H₂O₂-induced increase in DCF fluorescence. As shown previously (22), PDGF increased the amount of intracellular H₂O₂ in NIH 3T3 cells (Fig. 7C). This effect of PDGF was inhibited in cells overexpressing WT but not in those overexpressing C47S, consistent with a peroxidase function of 1-Cys Prx in vivo.

**Subcellular Localization of 1-Cys Prx**—The subcellular localization of 1-Cys Prx was investigated by immunoblot analysis of nuclear, organelle, cytosolic, and membrane fractions of A431 cells (Fig. 8). The lysosomal enzyme catalase and the nuclear protein histone were chosen as markers for the corresponding subcellular fractions. 1-Cys Prx was detected only in the cytosolic fraction.

![Figure 6](image6.png) **Fig. 6. Evaluation of Trx and GSH as electron donors to 1-Cys Prx.** A, GS protection activity of 1-Cys Prx, TPx II, and GPx in the presence of DTT, Trx, or GSH. The 25-μl reaction mixtures contained 50 mM Hepes-NaOH (pH 7.0), 0.5 μM of GS, 10 mM ascrobate (ascorbate was omitted when DTT was added as electron donor), 5 μM FeCl₃, one of three peroxidases (1-Cys Prx, TPx II, or GPx), and one of three electron donor systems (10 mM DTT; the Trx system, consisting of 3 μM Trx, 0.5 μM Trx reductase, and 0.4 mM NADPH; or the GSH system, consisting of 1 mM GSH, 1.2 units of glutathione reductase, and 0.4 mM NADPH). The concentration of peroxidase in the assay mixture was 3.8 μM for 1-Cys Prx, 3.6 μM for TPx II, and 0.1 μM for GPx in the presence of DTT; 16 μM for 1-Cys Prx and 0.55 μM for TPx II in the presence of the Trx system; and 16 μM for 1-Cys Prx and 0.1 μM for GPx in the presence of the GSH system. After incubation at 37 °C for 10 min, the remaining GS activity was measured as described (17). The extent of protection is expressed as a percentage relative to the inactivation apparent in the absence of peroxidase. B, NADPH oxidation coupled by 1-Cys Prx (dotted line) or TPx II (solid line) to the reduction of H₂O₂ in the presence of Trx and Trx reductase. NADPH oxidation was monitored as the decrease in A₃₄₀ at 37 °C in a 150-μl reaction mixture containing 50 mM Hepes-NaOH (pH 7.0), 250 μM NADPH, 46 mM Trx reductase, 6.7 μM Trx, 0.5 mM H₂O₂, and either 16 μM 1-Cys Prx or 1.07 μM TPx II. Data are representative of three similar experiments.

![Figure 7](image7.png) **Fig. 7. Peroxidase activity of WT and C47S 1-Cys Prx proteins in transfected cells.** A, NIH 3T3 cells were transiently transfected with the indicated expression plasmids (pCR represents the empty pCR3.1-Uni vector), and the extent of 1-Cys Prx expression was measured by immunoblot analysis. B and C, relative DCF fluorescence intensity/cell was measured by confocal microscopy after incubation of the transfected cells for 5 min with 10 μM H₂O₂ (B) or for 10 min with PDGF-AB (5 ng/ml) (C). Data in B and C are means ± S.E. of the values obtained from five groups of 10–20 cells. Data are means ± S.E. of three similar experiments.
and mutant proteins could be partially purified by collecting unbound proteins after mixing the cytosolic fraction of transfected NIH 3T3 cells with this resin. Expression of the various 1-Cys Prx proteins was confirmed with the unbound proteins by immunoblot analysis (Fig. 9A). The unbound proteins were also assayed for PLA2 activity at pH 4.0 in the absence of Ca2+. Expression of WT, C47S, C91S, S32A, or S32G was associated with a marked increase in Ca2+-independent PLA2 activity (Fig. 9B), suggesting that neither the two cysteines nor Ser32 directly participates in PLA2 catalysis.

**DISCUSSION**

Most peroxidases, including cytochrome c peroxidase, contain heme rings at their active sites. However, other peroxidases contain a redox-sensitive moiety such as selenocysteine (GPx (16)), vanadium (algal bromoperoxidase (23)), or flavin (bacterial NADH peroxidase (24)). TPx was the first peroxidase shown to contain no redox-sensitive moiety other than cysteine. The amino acid sequence identity among the four (one yeast and three mammalian) known TPx enzymes is >65%, with the homology being especially marked in the regions surrounding the two conserved cysteine residues that correspond to Cys47 and Cys170 of yeast TPx. The sequence identity among the seven 1-Cys Prx family members is >60% (Fig. 1), whereas that between human 1-Cys Prx and TPx enzymes is <30%. The consensus sequence surrounding the conserved cysteine of 1-Cys Prx proteins, which corresponds to Cys47 of human 1-Cys Prx, is PVCTTE and differs from the corresponding consensus sequence, FVCPTTE, of TPx enzymes. In addition to the cysteine corresponding to Cys47 of human 1-Cys Prx, some 1-Cys Prx members contain other cysteine residues, such as Cys91 of the human enzyme. However, neither Cys91 itself nor the sequence surrounding this residue is conserved among the 1-Cys Prx members (Fig. 1).

Our data now demonstrate that 1-Cys Prx is capable of removing H2O2 both in vitro and in vivo. In Fig. 10, the catalytic mechanism of 1-Cys Prx is compared with those of other peroxidases (TPx, GPx, and NADH peroxidase) that contain a cysteine or selenocysteine as the primary site of reaction with peroxides. Our experiments with cysteine mutants suggest that Cys47–SH is the site of oxidation in 1-Cys Prx. The oxidized products of cysteine include sulfenic acid, disulfide, sulfenic acid, and sulfonic acid (–SO3H). The disulfide with Cys47–SH, is as active as WT, and that Cys 91⋯SH reacts with the oxidized product of Cys47⋯SH only after denaturation. The fact that the oxidized product can be reduced back to cysteine by DTT excludes sulfenic and sulfonic acids as the intermediate (19). Alkyl sulfenic acids such as cysteine sulfenic acid are highly unstable and readily undergo condensation with thiols to produce disulfides (19). However, it appears that Cys91⋯SH is not sufficiently close to allow the formation of a disulfide with Cys47⋯SOH in the native form of oxidized human 1-Cys Prx. Recently, the existence of Cys-SOH has been conclusively demonstrated in the x-ray crystal structure of the oxidized native 1-Cys Prx.3 In contrast, in yeast TPx, Cys47⋯SOH reacts immediately with Cys170⋯SH of the other subunit of the homodimer to form an intermolecular disulfide that is subsequently reduced by electrons donated by Trx (Fig. 10B). An identical mechanism involving an intermolecular disulfide and reduction by Trx underlies the function of mammalian TPx enzymes (10). Kinetic studies on H2O2 reduction catalyzed by mammalian TPx I, II, and III have revealed the K_m for Trx to be ~3–6 μM, suggesting that TPx and Trx interact with a high affinity. The cysteine sulfenic acid of 1-Cys Prx and disulfide of TPx can be reduced to the thiol by DTT in a process involving two electrons. However, Trx was not able to reduce oxidized 1-Cys Prx.

Another well characterized reaction of sulfenic acid is its rapid oxidation by H2O2 to sulfenic and sulfonic acids (25). Thus, during the DTT-supported catalytic cycle of 1-Cys Prx, two reactions, reduction by DTT and further oxidation by H2O2, compete for the available Cys⋯SOH. Because of the irreversible nature of such oxidation, 1-Cys Prx eventually becomes inactivated in the presence of H2O2. The rate of inactivation depends on the concentration of H2O2. In cells, the extent of such inactivation is probably negligible, given that the cellular concentration of H2O2 is low and that reduction by the as yet unidentified physiological electron donor is likely to be much faster than that mediated by DTT. No substantial inactivation of TPx was detected at a low concentration of H2O2, probably because

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3 H.-J. Choi, S. W. Kang, C.-H. Yang, S. G. Rhee, and S. E. Ryu, unpublished results,
the primary site of catalysis, the proteases and protein-tyrosine phosphatases, in which cysteine is (Ka) un-ionized at neutral pH because of their higher pKa values. The sulfenic acid of TPx immediately forms an intermolecular disulfide and thereby escapes further oxidation by H2O2. However, in the presence of a higher concentration of H2O2, irreversibly inactivated TPx was apparent.

The reaction mechanism of 1-Cys Prx resembles that of the selenium-dependent GPx, which catalyzes peroxide reduction via the selenenic acid (Cys–SeOH) form of the selenocysteine (Cys–SeH) (26) (Fig. 10C). Unlike Cys–SeH, which is fully ionized to selenolate (Cys–Se-) at neutral pH and consequently readily reacts with H2O2, most proteinaceous cysteines remain un-ionized at neutral pH because of their higher pKa values (>8.5). However, in several enzymes, including cysteine proteases and protein-tyrosine phosphatases, in which cysteine is the primary site of catalysis, the pKa of the thiol is reduced to <7 as a result of electrostatic interaction between cysteine thiolate (Cys–S-) and basic residues (27, 28). The pKa of Cys47-SH of 1-Cys Prx is also expected to be decreased given its rapid reaction with H2O2 at pH 7. The sulfenate (Cys47–SO-) of the oxidized enzyme is also probably stabilized by the same basic residues that interact with the thiolate.

The selenenic acid of GPx readily reacts with GSH (as well as with the nonphysiological donor DTT) to form selenadisulfide (Cys-Se-SG), from which Cys-SeH is regenerated by means of a second GSH molecule (Fig. 10C). In contrast, the sulfenic acid of 1-Cys Prx is reduced by DTT but not by GSH. Because all thiols should reduce the sulfenic acid of 1-Cys Prx if it is accessible, the active site pocket of the enzyme may be large enough for H2O2 or DTT but not for two molecules of GSH. This notion is consistent with the observation that DTTB reacts with Cys49-SH but not with Cys47-SH in the native enzyme. Alternatively, a wrongly oriented GSH moiety shielding the active site from further approach by the second GSH in the crowded pocket would have a similar effect on GSH sensitivity.

Human 1-Cys Prx also resembles streptococcal NADH peroxidase in that a reversible conversion of the cysteine sulfenic acid is important in catalysis (24) (Fig. 10D). The sulfenic acid intermediate of NADH peroxidase is ensured by the fact that the active site cysteine is the only thiol of the enzyme. NADH peroxidase contains a stoichiometric amount of FAD that stabilizes thiolate and sulfenate anions in the reduced and oxidized forms of the enzyme, respectively, by forming a charge complex with them (24, 29). Furthermore, the streptococcal peroxidase binds NADH with high affinity, and the nucleotide provides electrons needed to reduce sulfenic acid via FAD. These characteristics distinguish the streptococcal peroxidase from 1-Cys Prx, which neither contains flavin nor utilizes nicotinamide nucleotides as an electron donor.

Amino acid sequencing of tryptic peptides derived from purified rat lung Ca2+-independent PLA2 revealed complete identity to the deduced amino acid sequence of human 1-Cys Prx. Furthermore, translation of mRNA derived from the human 1-Cys Prx (HA0683) clone in a wheat germ system resulted in expression of Ca2+-independent PLA2 activity (11). Although we failed to detect PLA2 activity with E. coli-expressed recombinant 1-Cys Prx, expression of the human 1-Cys Prx in NIH 3T3 cells was associated with an increase in PLA2 activity with properties similar to those of the activity shown by the rat lung enzyme. Because the deduced sequence of human 1-Cys Prx contains a motif, Gly-X-Ser32-X-Gly, associated with the catalytic site of a serine hydrolase, Ser32 was proposed to be the primary site of catalysis (11). Moreover, because the Ca2+-independent PLA2 activity was optimal at pH 4 and negligible above pH 6, the enzyme was presumed to be a lysosomal protein (11). The specific PLA2 activity measured at the optimal pH was only 40 nmol/min/mg of protein (estimated from Table I and Fig. 2 of Ref. 11). We have now shown that Ser32 is not required for Ca2+-independent PLA2 activity of 1-Cys Prx expressed in NIH 3T3 cells. This observation is consistent with the fact that the Gly-X-Ser-X-Gly motif is not conserved among 1-Cys Prx members (Fig. 1). Our data also suggest that 1-Cys Prx is not a lysosomal protein but is localized to the cytosol, the pH of which would be expected to prevent substantial manifestation of Ca2+-independent PLA2 activity.

It is not yet possible to estimate the specific peroxidase activity of 1-Cys Prx, because its physiological electron donor is not known. Nevertheless, the peroxidase activity of 1-Cys Prx measured in the presence of DTT is 2–3 times that of TPx II. The specific activity of TPx II was 3 μmol/min/mg of protein when measured in the presence of the Trx system (10). Thus, the peroxidase activity of human 1-Cys Prx is likely 2 orders of magnitude greater than the PLA2 activity at pH 4. More importantly, we demonstrated a peroxidase function for human 1-Cys Prx expressed in NIH 3T3 cells. Although H2O2 is generally considered a toxic by-product of respiration,
recent evidence suggests that the production of H$_2$O$_2$ might be an integral component of membrane receptor signaling. In mammalian cells, various extracellular stimuli, including cytokines and growth factors, induce a transient increase in the intracellular concentration of H$_2$O$_2$ (18, 22, 30). H$_2$O$_2$ thus generated is known to serve as a messenger that initiates various cellular responses including protein phosphorylation, NF-$\kappa$B activation, and apoptosis (22, 30, 31). To date, catalase and GPx have been viewed as the major enzymes responsible for the removal of cytotoxic H$_2$O$_2$. Recently, TPx enzymes were shown to be able to remove intracellular H$_2$O$_2$ generated in response to various extracellular stimuli, blocking the H$_2$O$_2$-mediated NF-$\kappa$B activation and apoptosis (10). Our data now suggest that 1-Cys Prx might also play a role in H$_2$O$_2$ removal.

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