Identification of a New Type of Mammalian Peroxiredoxin That Forms an Intramolecular Disulfide as a Reaction Intermediate

Running title: Characterization of mammalian PrxV

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SUMMARY

Peroxidases of the peroxiredoxin (Prx) family contain a Cys residue that is preceded by a conserved sequence in the NH₂-terminal region. A new type of mammalian Prx, designated PrxV, has now been identified as the result of a database search with this conserved Cys-containing sequence. The 162-amino acid PrxV shares only ~10% sequence identity with previously identified mammalian Prx enzymes and contains Cys residues at positions 73 and 152 in addition to that (Cys⁴⁸) corresponding to the conserved Cys. Analysis of mutant human PrxV proteins in which each of these three Cys residues was individually replaced with serine suggested that the sulfhydryl group of Cys⁴⁸ is the site of oxidation by peroxides, and that oxidized Cys⁴⁸ reacts with the sulfhydryl group of Cys¹⁵² to form an intramolecular disulfide linkage. The oxidized intermediate of PrxV is thus distinct from those of other Prx enzymes, which form either an intermolecular disulfide or a sulfenic acid intermediate. The disulfide formed by PrxV is reduced by thioredoxin, but not by glutaredoxin or glutathione. Thus, PrxV mutants lacking Cys⁴⁸ or Cys¹⁵² showed no detectable thioredoxin-dependent peroxidase activity, whereas mutation of Cys⁷³ had no effect on activity. Immunoblot analysis revealed that PrxV is widely expressed in rat tissues and cultured mammalian cells, and is localized intracellularly to cytosol, mitochondria, and peroxisomes. The peroxidase function of PrxV in vivo was demonstrated by the observations that transient expression of the wild-type protein, but not that of the Cys⁴⁸ mutant, in NIH 3T3 cells inhibited H₂O₂ accumulation and activation of c-Jun NH₂-terminal kinase induced by tumor necrosis factor-α.
INTRODUCTION

Peroxidases of the peroxiredoxin (Prx)\(^1\) family reduce hydrogen peroxide and alkyl hydroperoxides to water and alcohol, respectively, with the use of reducing equivalents derived specifically from thiol-containing donor molecules such as thioredoxin (Trx) (1,2), AhpF (3,4), and trypanothione (5,6). More than 40 members of this family have been identified in a variety of organisms from bacteria to plants to mammals (7). All Prx proteins contain a conserved cysteine residue in the NH\(_2\)-terminal portion of the molecule, and most contain an additional conserved Cys in the COOH-terminal region. The COOH-terminal and NH\(_2\)-terminal Cys residues are separated by 120 to 123 amino acids in Prx enzymes from bacteria, yeast, plants, and mammals, and the sequences surrounding each of these Cys residues are also highly conserved. A small number of Prx proteins, with representatives from most phyla, lack the COOH-terminal Cys (8). Members of the Prx family can thus be divided into two subgroups: 2-Cys Prx proteins, which contain both the NH\(_2\)- and COOH-terminal Cys residues, and 1-Cys Prx proteins, which contain only the NH\(_2\)-terminal Cys (8). Members of the 2-Cys Prx subgroup include four mammalian Prx enzymes, PrxI to PrxIV (9-12), which are the products of distinct genes in both humans and mice. In contrast, only one human and mouse 1-Cys Prx has been identified (8).

The 2-Cys and 1-Cys Prx enzymes exist as homodimers, with the two monomers oriented in a head-to-tail manner (13-15). The reaction mechanisms by which 2-Cys and 1-Cys Prx enzymes remove peroxides are distinct (8). In the case of 2-Cys Prx, peroxides oxidize the NH\(_2\)-
terminal Cys thiol group (Cys-SH) to sulfenic acid (Cys-SOH), which immediately reacts with the COOH-terminal Cys-SH of the other subunit to form an intermolecular disulfide. This disulfide is subsequently reduced specifically by Trx. Thus, mutant 2-Cys Prx proteins that lack either the NH$_2$-terminal or COOH-terminal Cys residues do not exhibit Trx-coupled peroxidase activity. The NH$_2$-terminal Cys-SH of 1-Cys Prx is also the site of oxidation by peroxides. However, the resulting Cys-SOH does not form a disulfide because of the unavailability of another Cys-SH nearby: Whereas the NH$_2$-terminal Cys is the only Cys residue in the entire molecule for the mouse 1-Cys Prx, human 1-Cys Prx does contain an additional Cys; however, mutational analysis indicated that this latter residue does not participate in the peroxidase reaction (8). The presence of Cys-SOH in the oxidized human 1-Cys Prx was demonstrated by determination of the crystal structure of the protein (14). The Cys-SOH of oxidized 1-Cys Prx can be reduced by nonphysiological thiols such as dithiothreitol (DTT) but not by Trx (8). The physiological electron donor (or donors) that supports the peroxidase activity of 1-Cys Prx remains to be identified.

We now describe the identification and characterization of a new type of mammalian Prx, which forms a reaction intermediate distinct from those of 2-Cys and 1-Cys Prx enzymes. This new Prx contains the conserved NH$_2$-terminal Cys as well as two additional Cys residues, neither of which, on the basis of the sequences surrounding them and their distances from the NH$_2$-terminal Cys, corresponds to the conserved COOH-terminal Cys of other members of the Prx family. Thus, this newly identified Prx resembles 1-Cys Prx enzymes in its primary structure. However, unlike 1-Cys Prx, the new enzyme forms an intramolecular disulfide intermediate,
which is also distinct from the intermolecular intermediate of 2-Cys Prx.
EXPERIMENTAL PROCEDURES

Materials—Glutathione reductase was obtained from Boehringer Mannheim. Trx, Trx reductase (TrxR), and glutaredoxin (Grx) were prepared as previously described (9). Glutamine synthetase was purified from Escherichia coli as described (1). Tumor necrosis factor-α (TNF-α) and platelet-derived growth factor (PDGF)-AB were obtained from Life Technologies and Upstate Biotechnology, respectively. Rabbit polyclonal antibodies to recombinant PrxV were produced by standard immunization procedures. An E. coli expression plasmid encoding a glutathione S-transferase (GST) fusion protein of c-Jun, a plasmid encoding hemagglutinin epitope (HA)-tagged c-Jun NH₂-terminal kinase (JNK), and peroxisomes isolated from guinea pig liver were kindly provided by N. Holbrook (National Institute of Aging, NIH), J. M. Kyriakis (Harvard Medical School), and A. Hajra (Department of Biological Chemistry, University of Michigan), respectively. Antibodies to PrxII and PrxIII were prepared as described (9).

Cloning and mutation of PrxV—Searches of a nonredundant database of human expressed sequence tags (ESTs) were performed with TBLastN 2.0 (16). For cloning of the human PrxV cDNA, we performed the polymerase chain reaction (PCR) with a human liver cDNA library as template, the forward primer 5’-ATCATATGGCCCAATCAAGGTGGGAGAT-3’, and the reverse primer 5’-TAGAATTCAGAGCTGAGATCATATGG-3’ (initiation and stop codons are indicated in bold). The forward and reverse primers contain Nde I and Eco RI sites, respectively, at their 5’ ends. The PCR product was directly ligated into the mammalian expression vector pCR3.1
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(Invitrogen), yielding pCRWT. The nucleotide sequence of the PrxV coding region was determined by standard automated procedures.

Three mutant human PrxV proteins, C48S, C73S, and C152S, in which Cys^{48}, Cys^{73}, and Cys^{152} were individually replaced by serine, were generated by standard PCR-mediated site-directed mutagenesis with pCRWT as template and complementary primers containing a single-base mismatch that converted the codon for Cys to one for Ser. The final mutated PCR products were also ligated into the pCR3.1 vector, yielding pCRC48S, pCRC73S, and pCRC152S, respectively. For expression of the wild-type and mutant proteins in *E. coli*, the Nde I-Eco RI fragments of pCRWT and the mutant plasmids were subcloned into the expression vector pET17b (Novagen), thereby generating pETWT, pETC48S, pETC73S, and pETC152S.

*Expression and purification of PrxV*—Cells of the *E. coli* strain BL21(DE3) pLysS (Novagen) were transformed with the pETWT plasmid, 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 to 0.8, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.4 mM. After incubation for an additional 3 h, the cells were collected by centrifugation, frozen in liquid nitrogen, and stored at –70°C until use. The recombinant PrxV protein was present in the soluble fraction of the bacterial cells (data not shown) and was monitored during purification by SDS-polyacrylamide gel electrophoresis (PAGE) and staining with Coomassie blue.

Frozen cells (4 g) were suspended in 20 ml of buffer A [20 mM Hepes-NaOH (pH 7.0), 1
mM EDTA] containing 2 mM DTT and were disrupted by pressure, and the resulting cell extract was centrifuged at 12,000 × g for 30 min. Streptomycin sulfate was added to the resulting supernatant to a final concentration of 1% (w/v), 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 until 80% saturation was achieved, after which the mixture was stirred for an additional 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 2 mM DTT and 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 (HPLC) on a TSK phenyl 5PW column (21.5 by 150 mm) that had been equilibrated with buffer A containing 1.5 M (NH₄)₂SO₄. Proteins were eluted with a decreasing gradient of ammonium sulfate from 1.5 to 0 M over 60 min at a flow rate of 5 ml/min. Fractions of 5 ml were collected, and those (fractions 28 to 32) corresponding to the peak of PrxV were pooled, dialyzed against 2 liters of buffer B [20 mM Tris-HCl (pH 7.5), 1 mM EDTA], and concentrated in an Amicon concentrator. The concentrated sample was reduced with 2 mM DTT for 10 min and applied to a Mono Q HR10/10 column (Pharmacia) that had been equilibrated with buffer B. The column was washed with the same buffer for 10 min. PrxV was detected in the flow-through material, and those fractions containing the protein were pooled, dialyzed against 2 liters of buffer A, and stored at −70°C until use. The mutant C48S, C73S, and C152S proteins were prepared by a procedure similar to that used for the wild-type enzyme.

Assay of PrxV activity—The ability of PrxV to protect glutamine synthetase from...
oxidative inactivation was measured as described previously (1), with a slight modification. The 25-µl reaction mixture, containing 0.5 µg of glutamine synthetase, 10 mM DTT, 3 µM FeCl₃, 50 mM Hepes-NaOH (pH 7.0), and various concentrations of PrxV, was incubated at 37°C for 10 min, after which 1 ml of γ-glutamyltransferase assay mixture was added and the remaining activity of glutamine synthetase was measured at 37°C for 3 min. The Trx-dependent peroxidase activity of PrxV was measured as described (17).

Subcellular fractionation—HeLa cells that had been grown to confluence in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum were washed twice with ice-cold phosphate-buffered saline. The washed cells were disrupted in a homogenization buffer [10 mM triethanolamine, 10 mM acetic acid (pH 7.4), 250 mM sucrose, 1 mM EDTA, 1 mM DTT, and aprotinin, leupeptin, pepstatin, and chymostatin each at a concentration of 10 µg/ml] by passing them 10 times through a 25-guage needle, followed by homogenization in a Dounce homogenizer. Nuclei and unbroken cells were removed by centrifugation of the homogenate at 1000 × g for 10 min. The resulting supernatant was further centrifuged sequentially at 14,000 × g for 30 min to separate organelles and at 100,000 × g for 1 hr to obtain plasma membrane and cytosolic fractions.

Transfection—NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, penicillin (100 U/ml), and streptomycin (100 U/ml), and were continuously passaged for 3 months after thawing. For transfection, the cells were plated at a density of 3 × 10⁵ per 60-mm dish, allowed to recover for 24 h, and then transfected with the indicated plasmids with the use of Superfect (Qiagen). After 24 h, the cells were deprived of
serum by incubation for an additional 18 h in the presence of 0.5% calf serum and then subjected to experiments.
RESULTS

Cloning and purification of recombinant PrxV—The amino acid sequence identity among the four human 2-Cys Prx (PrxI to PrxIV) enzymes is >70%, with the homology being especially marked in the regions surrounding the conserved NH2- and COOH-terminal Cys residues that correspond to Cys52 and Cys173 of PrxI (Fig. 1). The human 1-Cys Prx shares ~10% amino acid sequence identity with human 2-Cys Prx enzymes, but the sequence surrounding its NH2-terminal Cys (Cys47) is highly homologous to those surrounding the corresponding Cys of 2-Cys Prx enzymes (Fig. 1).

In an attempt to identify new Prx enzymes, we searched a database of human ESTs for sequences homologous to the NH2-terminal conserved sequence (KGKYVVLFFYPLDFTFVCP) of 2-Cys Prx enzymes. A human EST clone (GenBank accession number, H26194) with a Cys-containing sequence (KGKKGVLFVPGACFTPGCS) that shares 52% identity (indicated in bold) with the search sequence was thus detected. With the use of the nucleotide sequence of this clone, 87 EST clones containing identical overlapping sequences were further identified. We aligned all of these sequences in advanced BLAST searches and then constructed a 767-bp nucleotide sequence that includes a Kozak consensus sequence for translation initiation, an open reading frame encoding a 162-amino acid polypeptide, a stop codon (TGA) in the same frame, and a 135-bp 3' untranslated sequence containing a poly(A) tract. The newly identified putative Prx enzyme encoded by this nucleotide sequence was designated PrxV for reasons described below. Similar searches of a mouse EST database with the same NH2-terminal conserved
sequence of 2-Cys Prx enzymes also revealed a clone (GenBank accession number, AA472012) with a Cys-containing sequence that is identical to the corresponding portion of the human PrxV sequence. Identification of clones containing overlapping sequences yielded an open reading frame for a 162-amino acid protein that shows 95% sequence identity to human PrxV (Fig. 1).

Human PrxV is ~10% identical to human 2-Cys and 1-Cys Prx enzymes (Fig. 1). The COOH-terminal region of PrxV is smaller than those of 2-Cys Prx enzymes and lacks the conserved sequence containing the COOH-terminal Cys of the latter enzymes. Both human and mouse PrxV sequences contain Cys residues at positions 73 and 152 in addition to the conserved Cys48. However, the sequences surrounding Cys73 and Cys152 are not homologous to those surrounding the COOH-terminal conserved Cys residue of 2-Cys Prx enzymes, and the distances between Cys48 and these other two Cys residues are substantially smaller than the 120 to 123 residues that separate the two conserved Cys residues in typical 2-Cys Prx enzymes. PrxV shows 30% sequence identity to a *Saccharomyces cerevisiae* Prx known as type II Trx-dependent peroxidase (18) or peroxisomal membrane protein 20 (PMP20) (19) or alkyl hydroperoxide reductase (20); however, the yeast protein does not contain Cys residues corresponding to Cys73 and Cys152 of PrxV.

Human PrxV was expressed in *E. coli* and purified to homogeneity. The purified protein appeared as a single band with an apparent molecular size of 17 kDa on SDS-PAGE under reducing conditions (not shown), consistent with the size of 17,030 Da calculated from the predicted amino acid sequence. PrxV that had been oxidized with H2O2 was also detected as a monomer by SDS-PAGE under either reducing or nonreducing conditions (Fig. 2A), suggesting
that the protein does not form a disulfide-linked dimer on oxidation by H₂O₂. HPLC of PrxV on a gel filtration column in the presence of a buffer containing DTT yielded a peak at a position corresponding to 34 kDa (Fig. 2B), suggesting that PrxV exists as a dimer in its native state.

**Trx-dependent peroxidase activity of PrxV**—We investigated whether the reducing equivalents required for the presumed peroxidase activity of PrxV could be provided by the Trx system (Trx, TrxR, and NADPH) or the Grx system (Grx, GSH, GSH reductase, and NADPH). The rate of H₂O₂ degradation was measured by monitoring the decrease in A₃₄₀ attributable to the oxidation of NADPH. PrxV catalyzed the H₂O₂-dependent oxidation of NADPH in the presence of the Trx system (Fig. 3); the oxidation of NADPH required all three protein components (PrxV, Trx, and TrxR), being negligible in the absence of any one of the three. In contrast, the Grx system did not support the H₂O₂-dependent oxidation of NADPH by PrxV. Increasing the concentrations of Grx, GSH, and GSH reductase severalfold relative to those specified in the legend to Fig. 3 did not affect the inability of the Grx system to support the peroxidase activity of PrxV (data not shown). The functional efficacy of the Grx and GSH reductase preparations was demonstrated as described previously (21). These results suggest that PrxV receives reducing equivalents readily from Trx but not from Grx or from millimolar concentrations of GSH. PrxV also reduced t-butyl hydroperoxide in the presence of the Trx system with initial rates similar to that apparent for H₂O₂ reduction (data not shown).

Kinetic parameters for PrxV catalysis were determined by measuring the initial rates of NADPH oxidation at various concentrations of Trx and H₂O₂. Lineweaver-Burke plots (not shown) revealed that the Kₘ values of PrxV for Trx and H₂O₂ were 1 µM and <20 µM,
respectively, and that the $V_{\text{max}}$ at 37°C was 2 or 2.8 µmol/min per milligram of protein for the experiments in which the concentrations of Trx and H$_2$O$_2$, respectively, were varied.

**Peroxidase activity of Cys mutants of PrxV**—To study the catalytic role of the Cys residues of PrxV, we replaced each of the three residues at positions 48, 73, and 152 individually with serine, thereby generating C48S, C73S, and C152S mutant enzymes, respectively. The mutant proteins were expressed in *E. coli* and purified to homogeneity (Fig. 4A). Measurement of Trx-dependent peroxidase activity toward H$_2$O$_2$ revealed that the activity of the C73S mutant was similar to that of the wild-type enzyme, whereas no activity was detected with C48S and C152S proteins (Fig. 4B).

We also evaluated the peroxidase activities of the mutants by measuring their ability to protect glutamine synthetase from inactivation induced by a low concentration of H$_2$O$_2$ produced by a mixed-function oxidation system comprising O$_2$, DTT, and iron. In the presence of an electron donor such as DTT, iron catalyzes the reduction of O$_2$ to H$_2$O$_2$, which is further converted to hydroxyl radicals (OH$^*$) by the Fenton reaction (22). Glutamine synthetase possesses a binding site for divalent cations, at which bound iron catalyzes OH$^*$ production. The locally produced OH$^*$ results in oxidation and consequent inactivation of the enzyme (22). Given that oxidized Prx can be reduced by DTT, Prx prevents the inactivation of glutamine synthetase by the mixed-function oxidation system through removal of H$_2$O$_2$. We have previously used this glutamine synthetase protection assay for the detection of Prx activity, especially when we did not know the physiological source of the reducing equivalents (1,8). Consistent with the results of the Trx-dependent assay, C48S was completely inactive in the glutamine synthetase protection assay and the activity of C73S was equal to or slightly higher than that of wild-type PrxV (Fig.
4C). However, in contrast to the results obtained with the Trx-dependent assay, C152S protected glutamine synthetase, albeit less effectively than did the wild-type protein. These data suggest that Cys\textsuperscript{48} is essential for both Trx- and DTT-dependent peroxidase activities of PrxV, that Cys\textsuperscript{73} is not required for either of these two activities, and that Cys\textsuperscript{152} is essential for Trx-dependent activity but not for DTT-dependent activity.

**Formation of a disulfide bond between Cys\textsuperscript{48} and Cys\textsuperscript{152} of PrxV**—We next examined the possibility that Cys\textsuperscript{48} and Cys\textsuperscript{152} form a disulfide bond during the catalytic cycle of PrxV. PrxV that had been oxidized by H\textsubscript{2}O\textsubscript{2} was digested with endopeptidase Lys-C, and the resulting peptides were fractionated by HPLC on a C\textsubscript{18} column (Fig. 5, upper panel). Reduction of a portion of the Lys-C digest with DTT before HPLC resulted in the disappearance of peak I that was detected with the unreduced sample (not shown), suggesting that peak I likely contained peptides linked by a disulfide. Treatment with DTT of the manually collected peak I fraction followed by reinjection into the HPLC column yielded peaks II and III (Fig. 5, middle panel). Edman sequencing of the peptides corresponding to these latter two peaks yielded the sequences GVLFG and ALNVE, respectively, which match the five residues of the predicted Lys-C fragments containing Cys\textsuperscript{48} and Cys\textsuperscript{152}, respectively. We also prepared a Lys-C digest of oxidized PrxV that had been exposed to 5,5'-dithiobis-2-nitrobenzoic acid; fractionation of the digest by HPLC, with monitoring of elution of 5-thio-2-nitrobenzoic acid-labeled peptides by measurement of A\textsubscript{328}, revealed only one major labeled peak (peak IV) (Fig. 5, lower panel). The sequence of the peptide contained in peak IV was determined to be GVQVVA, which matches the first six residues of the Cys\textsuperscript{73}-containing Lys-C peptide of PrxV. These results indicate that oxidation of PrxV by H\textsubscript{2}O\textsubscript{2} results in the formation of a disulfide between Cys\textsuperscript{48} and Cys\textsuperscript{152}, whereas Cys\textsuperscript{73}-SH remains unoxidized.
Tissue, cellular, and subcellular distribution of PrxV—Total soluble fractions prepared from various rat tissues and cultured mammalian cells were subjected to immunoblot analysis with rabbit antibodies specific for PrxV (Fig. 6). Only one immunoreactive protein of 17 kDa was detected for all tissues and cells. Comparison of the blot intensities of this endogenous protein with those of various amounts of purified PrxV allowed us to estimate the amount of PrxV in micrograms per milligram of total soluble protein. The amounts of PrxV in rat tissues and cultured mammalian cells are shown together with those of other Prx enzymes in Tables I and II, respectively. Similar to other Prx isoforms, PrxV was expressed in almost all tissues and cell lines examined; it is especially abundant in kidney and kidney-derived KNRK cells, contributing as much as 0.13% of total soluble protein.

We next investigated the subcellular localization of PrxV by immunoblot analysis. HeLa cell homogenates were separated into organellar, plasma membrane, and cytosolic fractions. PrxV was detected in organellar and cytosolic fractions in a ratio of ~2:1 but was not detected in the plasma membrane fraction (Fig. 7A). The molecular size of PrxV detected in cytosolic and organellar fractions was identical at 17 kDa. Further fractionation of the organellar fraction on Nycodenz gradients (23) revealed that the distribution of PrxV was similar to that of the mitochondrial protein PrxIII, but not to that of the peroxisomal protein catalase, suggesting that most PrxV in the organellar fraction is present in mitochondria (data not shown). The presence of PrxV in peroxisomes was further investigated by immunoblot analysis of a peroxisomal fraction prepared from guinea pig liver. PrxV and catalase, but neither PrxIII nor the cytosolic enzyme PrxII, were detected in this fraction (Fig. 7B), which has been previously characterized by Webber and Hajra (24).
Peroxidase activity of PrxV in intact cells—Stimulation of NIH 3T3 cells with PDGF or TNF-α increases the intracellular concentration of H₂O₂, which can be monitored with the oxidation-sensitive fluorescent probe 2',7'-dichlorofluorescein diacetate and confocal microscopy. To determine whether PrxV is a physiologically relevant peroxidase in cells, we transiently transfected NIH 3T3 cells, which contain a relatively low amount of endogenous PrxV (Table II), with expression vectors encoding wild-type or C48S mutant proteins. Overexpression of the PrxV proteins was confirmed by immunoblot analysis (Fig. 8A). Exposure of cells transfected with the empty vector to TNF-α (15 ng/ml) for 10 min or PDGF (10 ng/ml) for 5 min resulted in 4.5- and 3.5-fold increases, respectively, in the fluorescence of 2',7'-dichlorofluorescein (DCF) (Fig. 8B). Expression of wild-type PrxV, but not that of C48S, markedly inhibited both the TNF-α- and PDGF-induced increases in DCF fluorescence.

The increase in intracellular H₂O₂ results in the activation of JNK (25,26). We therefore examined the effect of PrxV overexpression on the activation of HA-tagged JNK. TNF-α induced a twofold increase in JNK activity in NIH 3T3 cells that had been transfected with the empty PrxV vector, and this effect of TNF-α was partially inhibited by expression of wild-type PrxV, but not by expression of C48S (Fig. 9).
DISCUSSION

We have shown that PrxV catalyzes the reduction of $\text{H}_2\text{O}_2$ both in vitro and in vivo, and that Trx is likely the specific donor of the reducing equivalents required for the reduction reaction. Neither Grx nor GSH was able to support the peroxidase activity of PrxV. The simplest reaction mechanism for PrxV that is compatible with the observations that both Cys$^{48}$ and Cys$^{152}$ are essential for Trx-dependent activity and that the oxidized intermediate is a monomer containing the Cys$^{48}$-Cys$^{152}$ disulfide is shown in Fig. 10A. The C48S mutant of PrxV is inactive regardless of whether the reducing equivalents are provided by DTT or by Trx, whereas the C152S mutant is active in the presence of DTT but not in the presence of the Trx system. These results suggest that Cys$^{48}$ is the primary site of substrate peroxide reduction and is directly oxidized by $\text{H}_2\text{O}_2$ to yield $\text{H}_2\text{O}$ and Cys$^{48}$-SOH; the latter reacts with Cys$^{152}$-SH to form an intramolecular disulfide, which is subsequently reduced by Trx. In this model, Cys$^{48}$-SOH would react with one thiol of DTT to form a mixed disulfide in the absence of Cys$^{152}$, whereas the second thiol of DTT would attack the disulfide to produce Cys$^{48}$-SH and oxidized DTT (Fig. 10B). Such a mechanism would explain why the C152S mutant protects glutamine synthetase from oxidation by the DTT-containing mixed-function oxidation system, albeit with an efficiency lower than that of the wild-type protein.

The mechanisms of 2-Cys and 1-Cys Prx enzymes are shown in Fig. 10C and 10D, respectively. The four mammalian members (PrxI to PrxIV) of the 2-Cys Prx subgroup form intermediates that contain an intermolecular disulfide between the NH$_2$- and COOH-terminal Cys residues that correspond to Cys$^{52}$ and Cys$^{173}$, respectively, of human PrxI. The two disulfide-forming Cys residues of 2-Cys Prx enzymes are separated by 121amino acids, whereas
those of PrxV are separated by 104 residues. Moreover, the amino acid sequence surrounding Cys\textsuperscript{152} of PrxV does not resemble that surrounding Cys\textsuperscript{173} of PrxI. In human 1-Cys Prx, the NH\textsubscript{2}-terminal Cys (Cys\textsuperscript{47}) is the site of oxidation by H\textsubscript{2}O\textsubscript{2}, but the resulting Cys-SOH cannot form a disulfide because there is no other Cys-SH nearby. Although the physiological source of the reducing equivalents for the regeneration of Cys\textsuperscript{47}-SH is not known, DTT is able to support the regeneration in vitro. In this respect, the reaction mechanism of 1-Cys Prx resembles that of the C152S mutant of PrxV. The crystal structures of oxidized 1-Cys Prx (14) and PrxII (15) have revealed that both enzymes exist as head-to-tail dimers, in which the larger NH\textsubscript{2}-terminal domain of one subunit folds over the smaller COOH-terminal domain of the other subunit to form an active site. The presence of Cys-SOH in the active site of 1-Cys Prx and of an intermolecular disulfide in the active site of PrxII was apparent. The reactive Cys of 1-Cys Prx is located at the bottom of the active site pocket and is thus protected from larger oxidant molecules that contain a disulfide. Whether PrxV forms a similar head-to-tail dimer remains to be determined.

The catalytic efficiency of Prx V is similar to those of 2-Cys Prx enzymes: the $V_{\text{max}}$ of 2.0 to 2.8 $\mu$mol/min per milligram of protein for PrxV is smaller than those of 6 to 13 $\mu$mol/min per milligram of protein for Prx I, PrxII, and PrxIII, but the $K_m$ for Trx of 1 $\mu$M for PrxV is also smaller than those of 3 to 6 $\mu$M for the 2-Cys Prx enzymes (12). The $K_m$ for H\textsubscript{2}O\textsubscript{2} is <20 $\mu$M for PrxV and 2-Cys Prx enzymes. The catalytic efficiency of 1-Cys Prx has not been evaluated because its physiological donor of reducing equivalents is not known.

While the present study was in progress, PrxV was identified as proteins designated PMP20 and antioxidant enzyme B166 (AOEB166). Yamashita et al. (19) cloned human and
mouse PMP20 cDNAs as the result of a search for homologs of the yeast PMP20 protein, and Knoops et al. (27) cloned human and rat AOEB166 cDNAs as the result of an attempt to characterize a 17-kDa bronchoalveolar protein. Mammalian PMP20 proteins consist of 162 amino acids and contain a peroxisomal targeting sequence (Ser-Gln-Leu) at the COOH-terminus, as does PrxV (Fig. 1). The peroxisomal localization of PMP20 was demonstrated by expressing HA-tagged PMP20 in HeLa cells and immunostaining with antibodies to the HA tag. Knoops et al. observed that the AOEB166 cDNA contains two potential initiation sites in the same reading frame, the use of one of which would result in the production of a 162-residue protein identical to PrxV (PMP20), and the use of the other would generate a polypeptide of 214 residues. The 52 amino acid residues at the NH2-terminus of the longer polypeptide were shown to constitute a mitochondrial presequence that is capable of importing a fusion protein of AOEB166 and green fluorescent protein into mitochondria (27). Yamashita et al. (19) and Knoops et al. (27) demonstrated that the bacterially expressed 162-residue PMP20 (AOEB166) protein is able to protect glutamine synthetase from the DTT-containing mixed-function oxidation system.

Recognizing that AOEB166 is homologous (25 to 35% sequence identity) to yeast and bacterial members of the Prx family and that four mammalian Prx enzymes had been identified previously, Knoops et al. renamed the protein PrxV. Although PrxV is distantly related to Prx I to PrxIV (showing only 10% sequence identity), does not contain the COOH-terminal Cys conserved in 2-Cys Prx enzymes, and forms a distinct reaction intermediate, we believe that PrxV is an appropriate designation because the protein is a Trx-dependent enzyme whose function is dependent on two Cys residues.

With the use of immunoblot analysis with antibodies specific for PrxV, we estimated the amounts of PrxV in various rat tissues and cultured mammalian cells and compared them with
the amounts of Prx I, PrxII, PrxIII, and 1-Cys Prx. The relative abundance of PrxV protein among rat tissues was not in good agreement with the relative abundance of PMP20 or AOEB166 mRNAs as determined by Northern blot analysis. Like the other Prx enzymes, PrxV is widely expressed, with an abundance of 0.2 to 1.3 µg per milligram of soluble protein in the 13 rat tissues examined. Immunoblot analysis of subcellular fractions derived from HeLa cells and guinea pig liver revealed the presence of PrxV in cytosol, mitochondria, and peroxisomes, consistent with previous observations on the subcellular distribution of HA-tagged PMP20 and the AOEB166-green fluorescent protein fusion construct. The molecular size of PrxV in all tissues and cell lines examined as well as in the three subcellular fractions was identical to that of the 162-residue recombinant polypeptide. These observations, together with the results obtained with AOEB166, suggest that mitochondrial PrxV is synthesized in the cytosol from the first initiation site of PrxV mRNA as a 214-residue precursor protein and imported into mitochondria, where it is converted to the mature form with a size that is indistinguishable from that of the cytosolic and peroxisomal enzymes. Both of the latter are likely translated as 162-residue proteins from the second initiation site of PrxV mRNA.

Mitochondria and peroxisomes are the major source of H$_2$O$_2$ production in unstimulated mammalian cells. Molecular oxygen is reduced by electrons that leak from the mitochondrial respiratory chain to form the superoxide anion, which then undergoes spontaneous or enzyme-mediated dismutation to H$_2$O$_2$. Peroxisomes contain several enzymes that catalyze the oxidation of organic substrates such as fatty acids and D-amino acids and thereby generate H$_2$O$_2$. Mitochondria and peroxisomes are equipped with PrxIII and catalase, respectively, to protect against the toxic effects of H$_2$O$_2$. PrxV can now be added to the known antioxidant enzymes that
protect these two oxidant-generating organelles. As predicted from the long mitochondrial targeting sequence at its NH₂-terminus, PrxIII is synthesized in the cytosol as a preprotein that is converted to the mature form in mitochondria (28). The reducing equivalents required for the reactions of PrxIII and PrxV are likely provided by the recently discovered mitochondria-specific proteins Trx (29) and TrxR (30), both of which are also synthesized in the cytosol with mitochondrial targeting sequences. Whether peroxisomes also contain a specific Trx system for PrxV remains to be determined. PrxI, PrxII, and 1-Cys Prx are localized predominantly to the cytosol, and PrxIV, which contains a typical signal sequence of secretory proteins at its NH₂-terminus, is secreted outside of cells (11).

In many mammalian cell types, H₂O₂ is produced in response to a variety of extracellular stimuli that include TNF-α (31) and PDGF (32). This receptor-mediated generation of H₂O₂ in the cytoplasm has been linked to various intracellular signaling events such as the activation of mitogen-activated protein kinases (32) and the triggering of apoptosis (33,34). Specific inhibition of such H₂O₂ accumulation prevents these receptor-mediated signaling events. Overexpression of wild-type PrxV, but not that of the peroxidase-defective mutant C48S, inhibited H₂O₂ accumulation induced by TNF-α or PDGF as well as the TNF-α-induced activation of JNK in NIH 3T3 cells, suggesting that cytosolic PrxV likely participates in the signaling pathways of these extracellular stimuli.
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FOOTNOTES

1Abbreviations used are: Prx, peroxiredoxin; Trx, thioredoxin; DTT, dithiothreitol; TrxR, Trx reductase; Grx, glutaredoxin; TNF-α, tumor necrosis factor-α; PDGF, platelet-derived growth factor; GST, glutathione S-transferase; HA, hemagglutinin epitope; JNK, c-Jun NH2-terminal kinase; EST, expressed sequence tag; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; DCF, 2',7'-dichlorofluorescein.

2Glutathione has been suggested to be the physiological donor for 1-Cys Prx (35). However, we (8) and others (36) failed to detect GSH-supported peroxidase activity of 1-Cys-Prx.
FIGURE LEGENDS

Fig. 1. Alignment of the amino acid sequences of human PrxV (hPrxV) and mouse PrxV (mPrxV) with human 2-Cys (PrxI to PrxIV) and 1-Cys Prx proteins. The alignment was obtained by Clustal X (version 1.8) (37). Dashes within sequences represent gaps introduced to optimize alignment. Residue numbers are shown on the right. The positions of the NH$_2$-terminal Cys residue conserved in all Prx members (square), of the COOH-terminal Cys residue conserved in 2-Cys Prx enzymes (PrxI to PrxIV) (circle), and of Cys$^{73}$ and Cys$^{152}$ of PrxV (triangles) are indicated and boxed. The NH$_2$-terminal 52-residue sequence shown to include the mitochondrial targeting sequence of a PrxV preprotein (27) is not shown.

Fig. 2. SDS-PAGE and HPLC gel filtration analysis of recombinant human PrxV purified from E. coli. (A) Purified PrxV (10 µg in 20 µl) that had been oxidized with 0.1 mM H$_2$O$_2$ for 5 min was mixed with 20 µl of reducing sample buffer [125 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, 10 mM DTT] (lane 1) or nonreducing sample buffer (reducing sample buffer minus DTT) (lane 2), heated at 95°C for 5 min, subjected to SDS-PAGE on a 14% gel, and stained with Coomassie brilliant blue. The positions of molecular size markers (in kilodaltons) are indicated on the left. (B) Purified PrxV was applied to a Superose 12 PC 3.2/30 column (3.2 x 300 mm, Pharmacia) on a Pharmacia-LKB SMART System equipped with a µ separation
unit and a μ precision pump. The proteins were eluted at the flow rate of 40 μl/min with 20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl. Fractions of 40 μl were collected during monitoring by measurement of $A_{280}$. The peak positions of molecular size markers are indicated.

Fig. 3. NADPH oxidation coupled to the peroxidase activity of PrxV in the presence of the Trx or Grx systems. The initial rate of NADPH oxidation was monitored by measurement of the decrease in $A_{340}$ in the presence of PrxV at 37°C. The 150-μl reaction mixture contained 50 mM Hepes-NaOH (pH 7.0), 250 μM NADPH, 1.5 μM PrxV, and either 46 nM TrxR (squares), 2.2 μM Trx (triangles), both 46 nM TrxR and 2.2 μM Trx (closed circles), or the Grx system (46 nM GSH reductase, 2.2 μM Grx, 2 mM GSH) (open circles). The reaction was initiated by the addition of 0.5 mM H$_2$O$_2$.

Fig. 4. Effects of replacement of Cys$^{48}$, Cys$^{73}$, or Cys$^{152}$ of PrxV with serine on peroxidase activity. (A) Purified recombinant wild-type (WT) or mutant PrxV proteins (2 μg per lane) were treated with a reducing sample buffer containing 5 mM DTT and then analyzed by SDS-PAGE on a 14% gel. The positions of molecular size markers (in kilodaltons) are indicated on the left. (B) Initial rates of NADPH oxidation coupled to Trx-dependent peroxidase activity of PrxV enzymes were monitored by measurement of $A_{340}$ in a 150-μl reaction mixture containing 50 mM Hepes-NaOH (pH 7.0), 250 μM NADPH, 1.5 μM PrxV, 46 nM TrxR, and 2.2 μM Trx. Data are expressed as nanomoles of NADPH oxidized per minute. (C) Glutamine synthetase protection activities of the indicated concentrations of wild-type PrxV (triangles), C48S (squares), C73S
(circles), and C152S (diamonds) were measured as described in Experimental Procedures. Quantitative data in (B) and (C) are representative of three independent experiments.

Fig. 5. **Isolation of Cys-containing peptides from Lys-C digests of PrxV.** (Upper panel) Purified recombinant PrxV (200 µg) that had been oxidized with 0.1 mM H$_2$O$_2$ for 5 min was denatured with an unfolding buffer [6 M guanidine-HCl, 1 mM EDTA, 0.1 M potassium phosphate (pH 7.0)] and precipitated by 10% (w/v) trichloroacetic acid. The precipitate was resuspended in 300 µl of 10 mM Tris-HCl (pH 8.0) containing 10% (v/v) acetonitrile, and digested with Lys-C (4 µg) overnight. The resulting peptides were applied to an HPLC C$_{18}$ column, and were eluted with a linear gradient of 0 to 60% acetonitrile in 0.1% trifluoroacetic acid over 60 min. Elution was monitored by measurement of $A_{210}$. Peak I was collected. (Middle panel) HPLC analysis of peptides derived from peak I after reduction with DTT. (Lower panel) PrxV (200 µg) was oxidized with H$_2$O$_2$, labeled with 5-thio-2-nitrobenzoic acid by treatment with unfolding buffer containing 5 mM 5,5'-dithiobis-2-nitrobenzoic acid, and precipitated by 10% trichloroacetic acid. The precipitated protein was digested with Lys-C and analyzed by HPLC on the C$_{18}$ column. Elution was monitored by measurement of both $A_{210}$ and $A_{328}$.

Fig. 6. **Immunoblot analysis of PrxV expression in various rat tissues and cultured mammalian cells.** Total soluble fractions (30 µg of protein) of rat tissues (upper panel) or cultured mammalian cells (lower panel) were prepared as described (12) and subjected to SDS-PAGE on 14% gels. The separated proteins were transferred to a nitrocellulose membrane and probed with antibodies specific for PrxV. The first four lanes of each panel contain the indicated
amounts of purified PrxV. Immune complexes were visualized with the use of alkaline phosphatase-conjugated goat antibodies to rabbit immunoglobulin G. Cell lines examined included human epithelioid carcinoma HeLa cells, mouse NIH 3T3 fibroblasts, human epidermoid carcinoma A431 cells, rat embryonic thoracic aorta smooth muscle A10 cells, human chronic myelogenous leukemia K562 cells, human histiocytic lymphoma U937 cells, human Burkitt's lymphoma Ramos cells, human T cell leukemia Jurkat cells, human hepatocellular carcinoma HepG2 cells, rat thyroid FRTL cells, rat kidney KNRK cells, and rat adrenal pheochromocytoma PC12 cells.

Fig. 7. **Subcellular distribution of PrxV.** (A) The postnuclear supernatant (lane 1), organellar fraction (lane 2), plasma membrane fraction (lane 3), and cytosolic fraction (lane 4) of HeLa cells were subjected to immunoblot analysis with rabbit antibodies to PrxV. (B) Immunoblot analysis of a peroxisomal fraction of guinea pig liver with antibodies to either PrxV, Prx III, PrxII, or catalase. Lane 1 contains protein standards; lanes 2 to 4 contain 10, 20, and 40 µg, respectively, of peroxisomal proteins.

Fig. 8. **Effect of PrxV overexpression on H$_2$O$_2$ generation in response to TNF-α or PDGF-AB.** (A) NIH 3T3 cells were transiently transfected with 10 µg of the indicated PrxV expression plasmids (pCR represents the empty pCR3.1 vector), and the extent of PrxV expression was measured by immunoblot analysis of cell lysates (20 µg of protein). (B) The transfected cells were incubated either for 10 min with TNF-α (15 ng/ml) or for 5 min with PDGF-AB (10 ng/ml), after which relative DCF fluorescence intensity per cell was measured by confocal
microscopy (9). Data are means ± SE of values obtained from five random groups of 20 to 30 cells and are representative of three independent experiments.

Fig. 9. **Effect of PrxV overexpression on TNF-α-induced activation of JNK.** (A) NIH 3T3 cells were transiently cotransfected with a reporter plasmid encoding HA-JNK (3 µg) and the indicated PrxV expression plasmids (6 µg). Cells were stimulated with TNF-α (15 ng/ml) and the activity of JNK was assayed with GST-c-Jun as substrate as described (38). The top panel represents an autoradiogram showing the phosphorylation of GST-c-Jun. The middle and bottom panels represent immunoblot analysis of cell lysates with antibodies to JNK1 and Prx V, respectively. (B) The radioactivity associated with the GST-c-Jun bands in autoradiogram shown in the upper panel of (A) was quantitated by Phosphorimager analysis and expressed relative to the value for non-stimulated pCR3.1-transfected cells. Similar results were obtained from four independent experiments.

Fig. 10. **Comparison of the peroxidase reaction mechanisms.** Proposed reaction mechanisms of wild-type PrxV supported by Trx (A), the C152S mutant of PrxV supported by DTT (B), PrxI (representative of 2-Cys Prx enzymes) supported by Trx (C), and 1-Cys Prx supported by DTT or XH₂, the as yet unidentified electron donor for 1-Cys Prx (D), are shown. Closed circles indicate the NH₂-terminus of each protein.
Table I. Amounts of various Prx isoforms in the indicated rat tissues. Data are expressed as micrograms of Prx per milligram of soluble protein.

| Tissues     | Prx Va | Prx Ib | Prx IIb | Prx IIIb | 1-Cys Prx |
|-------------|--------|--------|---------|----------|-----------|
| Placenta    | 0.7    | 0.2    | 0.7     | <0.3     | 0.2       |
| Thymus      | 0.2    | 0.3    | 0.7     | 0.3      | 0.03      |
| Testicle    | 0.2    | 0.7    | 1.0     | 0.3      | 1.0       |
| Thyroid     | 0.3    | 0.7    | 0.7     | 0.5      | 0.2       |
| Pancreas    | 0.2    | N.D. d | N.D. d  | 0.3      | 0.03      |
| Adrenal     | 0.3    | >2.0   | 2.0     | >4.0     | 0.3       |
| Brain       | 1.0    | 1.3    | 1.3     | 0.5      | 1.7       |
| Hypothalamus| 0.7    | 1.3    | 1.3     | 0.5      | 0.7       |
| Spleen      | 0.3    | 0.7    | 2.0     | 0.3      | 0.03      |
| Lung        | 0.3    | 1.0    | 1.3     | 0.3      | 1.7       |
| Kidney      | 1.3    | 2.0    | 0.7     | 0.7      | 0.3       |
| Liver       | 0.7    | 0.7    | 0.5     | 0.7      | 0.3       |
| Heart       | 0.5    | 1.3    | 2.0     | 3.3      | 0.3       |

\( ^a \) Estimated from data in Fig.6.
\( ^b \) From ref.12.
\( ^c \) S.W. Kang and S.G. Rhee, unpublished work.
\( ^d \) N.D., not detectable.
Table II. Amounts of various Prx isoforms in the indicated cultured mammalian cells. Data are expressed as micrograms of Prx per milligram of soluble protein.

| Cell types | Prx I<sup>a</sup> | Prx II<sup>b</sup> | Prx III<sup>b</sup> | 1-Cys Prx<sup>c</sup> |
|------------|------------------|-----------------|-----------------|-----------------|
| HaLa       | 0.5              | >4.0            | 3.3             | 0.3             | >3.0            |
| NIH3T3     | 0.03             | 3.3             | 2.7             | 1.0             | 0.2             |
| A431       | 0.3              | 2.0             | 1.0             | <0.3            | 1.7             |
| A10        | N.D.<sup>d</sup> | <0.3            | 0.3             | 1.3             | N.D.<sup>d</sup> |
| K562       | 0.3              | 3.3             | 2.0             | <0.3            | >5.0            |
| U937       | 0.5              | 2.7             | 0.5             | <0.3            | 1.3             |
| Ramos      | 0.5              | 3.3             | 1.3             | <0.3            | 1.0             |
| Jurkat     | 0.5              | 2.7             | 1.0             | <0.3            | 1.0             |
| HepG2      | 0.5              | 3.3             | 1.3             | 0.5             | 1.7             |
| FRTL       | 0.3              | 1.3             | 1.3             | 3.3             | N.D.<sup>d</sup> |
| KNRK       | 1.3              | 2.1             | 1.0             | 0.7             | N.D.<sup>d</sup> |
| PC12       | 0.2              | 2.0             | 3.3             | 0.7             | 0.2             |

<sup>a</sup>Estimated from data in Fig.6.
<sup>b</sup>From ref.12.
<sup>c</sup>S.W.Kang and S.G.Rhee, unpublished work.
<sup>d</sup>N.D., not detectable.
Identification of a new type of mammalian peroxiredoxin that forms an intramolecular disulfide as a reaction intermediate
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