ENZYME CATALYSIS AND REGULATION:
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Cyclophilin A Binds to Peroxiredoxins and Activates Its Peroxidase Activity*

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Six distinct peroxiredoxin (Prx) proteins (Prx I-VI) from distinct genes have been identified in mammalian tissues. Prxs are members of a group of peroxidases that have conserved reactive cysteine residue(s) in the active site(s). An immediate physiological electron donor for the peroxidase catalysis for five Prx proteins (Prx I-V) has been identified as thioredoxin (Trx), but that for Prx VI (1-Cys Prx) is still unclear. To identify an immediate electron donor and a binding protein for Prx VI, we performed a Prx VI protein overlay assay. A 20-kDa binding protein was identified by the Prx VI protein overlay assay with flow-through fractions from a High-Q column with rat lung crude extracts. Using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) and MS-Fit, we identified the 20-kDa Prx VI-binding protein as a cyclophilin A (CyP-A). The binding of recombinant human CyP-A (hCyP-A) to Prx VI was confirmed by using the hCyP-A protein overlay assay and Western immunoblot analysis with hCyP-A-specific antibodies. hCyP-A enhanced the antioxidant activity of Prx VI, as well as the other known mammalian Prx isotypes. hCyP-A supported antioxidant activity of Prx II and Prx VI both against thiol (dithiothreitol)-containing metal-catalyzed oxidation (MCO) systems and ascorbate-containing MCO systems. Prx II was reduced by hCyP-A without help from any other reductant, and the reduction was cyclosporin A-independent. These results strongly suggest that CyP-A not only binds to Prx proteins but also supports its peroxidase activity as an immediate electron donor. In addition, Cys115 and Cys161 of hCyP-A were found to be involved in the activation and the reduction of Prx.

Peroxiredoxin (Prx) is a member of a growing family of thiol peroxidases that reduce peroxides to corresponding alcohols using reactive cysteine residue(s) in its active center (1). Recent searches of the public data bases show more than 100 members of the Prx family from archaeabacteria to human. All of the Prx family members that have been examined for their antioxidant capacity show a measurable protection of glutamine synthetase (GS) inactivation against oxidative insults (1–9). Their GS protection capability is caused by peroxide reducing activity in the presence of an appropriate electron donor such as thioredoxin (Trx). All Prx family members that contain two conserved cysteine residues (Prx I–IV) and one member containing one conserved cysteine residue (Prx V) have an H2O2 reducing activity that uses thioredoxin as an immediate electron donor and is thus named thioredoxin peroxidase (TPx) (9–12).

Recently, another member of the Prx family containing only one conserved cysteine residue (1-Cys Prx, later classified as a Prx VI) has been identified in several organisms (5, 13–18). In barley seeds, a functional relationship of this protein to dormancy has been reported (13); however, transgenic tobacco that overexpress 1-Cys Prx (Prx VI) of rice do not exhibit this correlation (14).

Glutathione peroxidase (GPx) and phospholipase A2, bifunctional enzymatic activities in recombinant human Prx VI have recently been confirmed (15–16). Recombinant Prx VI protein reduced hydrogen peroxide, as well as phospholipid hydroperoxides, in the presence of GSH (17). Bovine and mouse counterparts were reported to have a novel GPx activity that does not contain selenium at its active site (18–19). Although the GPx activity of Prx VI has been confirmed, peroxidase activity of Prx VI in the presence of GSH was generally much lower than that with dithiothreitol (DTT), while the GS protection activity of Prx VI in the presence of DTT was comparable with selenium-GPx as well as other Prx members (5). Thus, the presence of other physiological electron donors was suggested.

To identify the possible electron donor or the protein functionally linked to Prx VI, we performed a Prx VI protein overlay assay with fractionated rat lung crude extracts. We have now shown that cyclophilin A (CyP-A) binds to Prx VI and enhances its antioxidant activity. We also provide evidence that CyP-A can be a general electron donor for all known mammalian Prx isotypes.

**EXPERIMENTAL PROCEDURES**

Materials—GS was purified from Escherichia coli K12 as described previously (20). Recombinant human Prx proteins were purified from the E. coli overexpression system as previously described (4–5, 10). Polyclonal antiserum to Prx VI and human CyP-A (hCyP-A) were prepared from rabbits by injecting purified recombinant Prx VI and hCyP-A, respectively.

Nitrocellulose membrane (BA-85) was obtained from Schleicher and Schuell, and the alkaline phosphatase-labeled anti-rabbit IgG produced in goat was purchased from KPL Laboratories.
Fractionation of Rat Lung Crude Extract with High-Q Anion-Exchange Column Chromatography—Rat lungs (10 g) were homogenized with 20 ml of 20 mM Tris-HCl buffer (pH 7.6) containing 100 μg/ml of phenylmethylsulfonyl fluoride, and the homogenate was centrifuged for 30 min at 20,000 × g. The clear supernatant was applied to a High-Q strong anion-exchange column (Econo-pac, 5 ml, Bio-Rad) that had been equilibrated with 25 mM Tris-HCl (pH 7.6). After washing for 10 min with equilibration buffer, proteins were eluted with a linear NaCl gradient from 0 to 1 M for 200 min. The flow rate was 0.5 ml/min and 5-ml fractions were collected.

Overlay Assay—Proteins in the fractions eluted from the High-Q column were spotted onto an SDS-PAGE gel, and the proteins were visualized with Coo massie Brilliant Blue R-250. The stained protein band that corresponds to the position of the putative Prx VI-binding protein was excised and successively washed with 30% ethanol and 100% acetone. After drying the gel pieces in a SpeedVac (Savant) for 30 min, a rehydration and proteolysis was done by adding 12.5 μg/ml of trypsin at 37 °C for 12 h in 25 mM ammonium bicarbonate buffer (pH 8.0). Resulting tryptic peptides were analyzed using a MALDI-TOF mass spectrometer equipped with delayed ion extraction (VOYAGER-DE STR, PE Biosystems).

Bacterial Expression and Purification of Recombinant WT and Mutant CyP-A—cDNA encoding hCyP-A was amplified by polymerase chain reaction (PCR) from a U-937 histamine hysticoma human cell cDNA library. The primers for amplified hCyP-A were designed based on the reported human T-cell cyclophilin cDNA sequence (21). Primers for hCyP-A are as follows: 5'-CyP (5'-GCCCAT/G/H11032 TTGACCCGCAC-CGGTGTCC-3'), which contains an initiation codon (italicized) and an NdeI site (underlined), and 3'-CyP (3'-ACACCGTGTTGACCTACAC-CTAGG GTT-5'), which contains a stop codon (italicized) and a BamHI site (underlined). Four cysteine residues (Cys29, Cys52, Cys62, and Cys115) of hCyP-A were individually mutated to serine by recombinant E. coli BL21(DE3)pLysS was transformed with pET-17b-con- 
ed vector (C161S). The primers for cysteine to (italicized) and a NdeI site (underlined), and 3'-CyP (3'-ACACCGTGTTGACCTACAC-CTAGG GTT-5'), which contains a stop codon (italicized) and a BamHI site (underlined). Four cysteine residues (Cys29, Cys52, Cys62, and Cys115) of hCyP-A were individually mutated to serine by recombinant E. coli BL21(DE3)pLysS was transformed with pET-17b-con- 
ed vector (C161S). The primers for cysteine to

RESULTS

Fractionation of Rat Lung Crude Extract and Overlay of Prx VI—To search for the putative Prx VI-binding protein, we first tried the overlay assay on a protein blot prepared from rat lung crude extract after separation on an SDS-PAGE gel. Various protein bands representing potential Prx VI complexes were visualized on the blotted membrane; however, because of low resolution, it was not easy to identify the corresponding protein band on the Coo massie Blue-stained SDS-PAGE gel (data not shown). Sufficient resolution was acquired with a High-Q anion-exchange column chromatography (fraction 2-9) were separated on a 13.5% SDDS-PAGE gel and electrophoret in a nitrocellulose membrane. Prx VI was overlaid on the nitrocellulose membrane and reacting Prx VI complexes were visualized with Prx VI-specific antiserum as described under “Experimental Procedures.” Lane C contains 200 ng of purified recombinant Prx VI. C, all the procedures were the same as those used for panel B except for the Prx VI overlay. Lane S contains 300 ng of purified recombinant Prx VI. D, electroblotted nitrocellulose membrane was probed with hCyP-A-specific antiserum. Lane S contains 200 ng of purified recombinant hCyP-A.

with DTT) or 12.5 μM (with ascorbate) FeCl3, 50 mM Hepes-NaOH (pH 7.0), and Prx VI and/or hCyP-A was incubated at 30 °C for 10 min to inactivate GS. The remaining activity of GS was measured by adding a γ-glutamyltransferase assay mixture at 30 °C for 5 min. Absorbance resulting from the γ-glutamylhydroxamate-Fe3+ complex was measured at 540 nm.

Identification of the Prx VI-binding Protein by MALDI-TOF Mass Spectrometry.
Calculated tryptic peptide masses from rat labeled with the submitted for data base search. The peaks with the prominent 20-kDa Prx VI-binding protein. The mass map was produced with the eluted peptides after in-gel digestion of the 20-kDa protein band with trypsin. The mass of the peaks marked with triangles (either filled or blank) was submitted for data base search. The peaks labeled with the filled triangle match calculated tryptic peptide masses from rat cyclophilin A within 50 ppm.

Fig. 2. Peptide mass map of the prominent 20-kDa Prx VI-binding protein. The mass map was produced with the eluted peptides after in-gel digestion of the 20-kDa protein band with trypsin. The mass of the peaks marked with triangles (either filled or blank) was submitted for data base search. The peaks labeled with the filled triangle match calculated tryptic peptide masses from rat cyclophilin A within 50 ppm.

TOF—Among the three candidates (about 34, 27, and 20 kDa), we focused our efforts on the characterization of the smallest protein (20 kDa). After separation of the proteins in fraction number 4 (Fig. 1A) on a 12.5% SDS-PAGE gel, the protein band at around 20 kDa that corresponded to the position of a positive signal on the overlay assay was excised after Coomassie Blue staining. Proteins in the excised acrylamide gel were in-gel trypsinized, and the molecular weight of resulting peptides was analyzed by MALDI-TOF. We got nine possible peptide signals with masses (m/z) of 1251.7701, 1267.7807, 1274.8443, 1572.8253, 1612.8443, 1759.9830, 1831.9727, 1947.9883, and 1990.0351 (Fig. 2). A search of MS-Fit (algorithm by Peter Baker and Karl Clauser) with resulting peptide masses, offered four protein candidates originating from rat, human, and bovine. Profilin II, from the human obtained the highest MOWSE (molecular weight search) score (27.1) among four candidates. However because of the smaller size of profilin II (15 kDa) compared with the tentative Prx VI-binding protein (20 kDa) and the acidic pl of human profilin II (pI, 5.78), which renders this protein able to bind on an anion-exchange column, we could easily rule out human profilin II from the candidates. Other candidates, rat neuronal protein NP25 and bovine α-S2 casein precursor were disregarded because of the discrepancy in source, molecular size (24712.3 and 26018.9, respectively), and/or pl (6.53 and 8.54, respectively). Considering the source, molecular weight (17874.4), and the pl (8.34), rat cyclophilin A (CyP-A) was a possible candidate for the 20-kDa Prx VI-binding protein. Four of nine peptide masses were matched with the rat cyclophilin sequence; aa 134–144 and the same sequence with Met-ox, aa 77–91, and aa 1–19 with a modified N-acetyl group.

Confirmation of the 20-kDa Prx VI-binding Protein as a CyP-A—To verify the 20-kDa tentative Prx VI-binding protein as a CyP-A, electrophobotted nitrocellulose membrane containing proteins from High-Q fraction number 4 was probed with Prx VI-specific antibody after overlay with Prx VI protein (Fig. 3A, lane 4). A band of about 20 kDa was stained with the Prx VI-specific antibody (lane 4, marked as an arrow). The 20-kDa Prx VI overlay signal was closely matched with the hCyP-A-specific antibody immunoblot signal of a purified recombinant hCyP-A (Fig. 3A, lane 1) and of a High-Q column fraction 4 (Fig. 3, lane 2). In addition, the 20-kDa Prx VI overlay signals (Fig. 1B) paralleled the hCyP-A immunoblot signals of High-Q fractions (Fig. 1D). To reaffirm the binding of hCyP-A to Prx VI, a hCyP-A overlay was employed instead of the Prx VI overlay.

Prx VI was successfully recognized by the hCyP-A antiserum after overlay with hCyP-A (Fig. 3B, lane 4) whereas the non-overlaid control did not (Fig. 4, lane 2).

Enhancement of Prx VI Antioxidant Activity by hCyP-A—GS protection activity of Prx VI in the presence of hCyP-A was measured. GS protection activity of Prx VI (10 μg/ml) proportionally increased with the addition of hCyP-A to the assay mixture, up to 60 μg/ml. An addition of 100 μg/ml or higher concentrations of hCyP-A to the assay mixture did not further increase the antioxidant activity of Prx VI. The activation of Prx VI (10 μg/ml) by hCyP-A reached 40% of complete protection, which corresponded to the GS activity of 40 μg/ml of Prx VI alone. hCyP-A by itself did not show considerable GS protection activity, up to 400 μg/ml.

Activation of Other Prx Family Members by hCyP-A—hCyP-A-dependent activation of other human Prx isotypes was determined (Fig. 5). Basal GS protection activities were normalized by the Prx protein concentration (40 μg/ml for Prx I-IV and VI, 200 μg/ml for Prx V), which offered 20–40% of complete protection. GS protection activity of Prx isotypes increased from 1.8 to 3.4-fold by supplementing 200 μg/ml of hCyP-A to the assay mixture. The activity of Prx II and IV increased about 3.5-fold and that of Prx I, III, and VI increased by 2.2, 2.8, and 2.4-fold, respectively. The activation of Prx V was much lower (1.8-fold) than other isotypes, however increments of activity were consistent. Activation of Prx by CyP-A was species-specific. Neither 2-Cys Prx from budding and fission yeast, nor from E. coli, was activated by hCyP-A (data not shown).

Reduction of Prx by hCyP-A—GS protection activity of Prx II and VI against the thiol- and ascorbate-containing MCO systems was measured in the presence of hCyP-A (Fig. 6A). hCyP-A supports GS protection activity of Prx II and VI in the absence of a disulfide-reducing reagent such as DTT or 2-mercaptoethanol, suggesting the presence of an intrinsic Prx reducing activity of hCyP-A. To confirm whether the hCyP-A itself has the capacity to reduce Prx, we investigated the hCyP-A-dependent reduction of an intermolecular disulfide bond of Prx II by using non-reducing SDS-PAGE analysis. Because of the intermolecular disulfide bond, oxidized 2-Cys Prx (Prx I-IV) containing disulfide bond(s) banded at the dimeric range (around 45 kDa), whereas the fully reduced form banded at the monomeric range on non-reducing SDS-PAGE gel (22). Considerable reduction of Prx II was observed in the presence of 5 μg of hCyP-A (Fig. 6B, lane 2), whereas the addition of 100 μM DTT or 0.48 μg of Trx (not reduced) did not reduce Prx II.
considerably (Fig. 6B, lanes 3, 4). Trx (with DTT) reduced Prx II very efficiently (Fig. 6B, lane 6); however, a supplement of DTT (100 μM) to the hCyP-A did not allow for an observable change in the reduction of Prx II (Fig. 6B, lane 5). The thiol group-modified hCyP-A using N-ethylmaleimide (NEM), completely lost its Prx II reducing activity (Fig. 7B, lanes 4–5). The fact that NEM modification and the resulting inhibition of Prx II reduction were successful without prereduction (Fig. 7B, lane 4) suggests thiol groups, which involve the reduction of Prx, mostly exist in reduced states during purification and storage.

To investigate the potential role of cysteine residues of hCyP-A in Prx activation or reduction, we measured the stimulation of the GS protection activity of Prx II and the Prx II reduction activity of recombinant WT, C52S, C62S, C115S, and C161S hCyP-A. Cys115 and Cys 161 were responsible for both stimulation of the GS protection activity and the reduction of Prx (Fig. 8A and B). The addition of cyclosporin A (CsA) to an assay mixture did not cause any detectable differences, either in GS protection activity (data not shown) or hCyP-A-dependent Prx II reduction (Fig. 7A).

**DISCUSSION**

Using the Prx VI (1-Cys Prx) overlay assay, we have identified CyP-A as a Prx VI-binding protein and its activation of Prx VI antioxidant activity. We also have shown CyP-A-dependent enhancement of GS protection activity, using all the known human Prx isotypes. hCyP-A supported GS protection activity of Prx II and VI even against the ascorbate-containing MCO.
system, and hCyP-A-dependent reduction of Prx II was also revealed. Considering the GS protection activity of Prx, which comes from the intrinsic peroxidase activity of Prx (9) and the Prx reducing activity of CyP-A (Figs. 6–7), it is reasonable to speculate CyP-A as an immediate and a general electron donor for the peroxidase catalysis of Prx. Jaenschke et al. (23) reported the binding of hCyP-A to Aop 1 (Prx III) and hCyP-A-dependent stimulation of Aop 1 antioxidant activity (23). However the physiological meaning of that interaction is not certain, because the cellular location of Prx III is in the mitochondrial matrix and that of hCyP-A is cytosolic. Even though all known human Prx isotypes were activated with hCyP-A regardless of intracellular compartments, only the cytosolic Prx isotypes (Prx I, II, and IV) that show the potential for physical interaction with hCyP-A might have physiological relevance.

CyP was originally isolated as a CsA-binding protein, and later calcineurin inhibiting capability when complexed with CsA was revealed. CyPs also catalyze cis/trans interconversion of X-Pro epitopes in proteins (24). CsA blocks the peptidyl cis-trans-isomerase activity (PPIase activity) of CyPs by binding to the active site Trp122 residue of hCyP-A (25), whereas each of the four Cys residues was dispensable for the binding of CsA to hCyP-A and the PPIase activity of hCyP-A (26). Our results and a previous report (23), which investigated the GS protection activity of hCyP-A, are consistent with these findings.
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FIG. 8. Role of cysteine residues of hCyP-A on the activation and reduction of Prx II. A, glutamine synthetase protection activity of Prx II was measured in the presence of wild-type and various cysteine mutants (C52S, C62S, C115S, C161S) of hCyP-A. 40 µg/ml of Prx II and 200 µg/ml of each hCyP-A protein were used for the assay of glutamine synthetase protection activity. Other procedures were the same as described in the legend to Fig. 4 B. 100 ng of Prx II was incubated for 10 min at room temperature in the absence or presence of 400 ng of each of the hCyP-A proteins, and the reduction of Prx II was analyzed using non-reducing 12% SDS-PAGE and immunoblot analysis.

Among the four Cys residues (Cys52, Cys62, Cys115, Cys161) of hCyP-A, Cys115 and Cys161 were responsible for the reduction and the activation of Prx (Fig. 7). The distance between Cys115 and Cys161 is calculated to be 20.08 Å (SYBYL, Tripos, Inc.), and the two Cys residues are localized at opposite sides in the globular structure of hCyP-A (1VBS.pdb). Consequently, the formation of intramolecular disulfide linkage between Cys115 and Cys161 through the reduction of Prx is not assumed to be possible. Considering the exposed (Cys161) and partially buried (Cys115) status in the globular structure, intramolecular disulfide bond formation involving two Cys161 residues is feasible, but not between two Cys115 residues or Cys115–Cys161, unless considerable conformational changes occur. In addition, the possibility of either one of the two or both of the Cys residues oxidizing to sulfenic acid through sulfenic acid coupled to Prx reduction cannot be ignored.

Although various biological functions and activities have been reported with respect to Prx proteins such as an inhibition of apoptosis (40), an inhibition of c-Abl tyrosine kinase activity (41), a regulation of NK-κB and AP-1 (4, 42), and a peroxinitrite reductase activity (43), only the peroxidase activity is commonly accepted as the catalytic activity of Prx. More than a thousand times higher affinity (<10 µM) to H2O2 than that of catalase (25 mM) (10, 44), inhibition of the peroxidase activity of Prx with a relatively higher (>100 µM) concentration of peroxide,2 and an abundance of Prx proteins in various cells or tissues (44), lead us to assume that Prx proteins might be functioning to remove particularly low concentrations of peroxide that are produced as a second messenger. Recently, growing evidence suggests a transient and modicum generation of hydrogen peroxide as part of membrane receptor signaling (reviewed in Ref. 45). A report that demonstrates the capacity of Prx, which removes hydrogen peroxide generated in growth signaling (46), supports this assumption. The detailed role of isotype-specific Prx/CyP-A complex in the membrane-receptor signaling as one of the possible signal modulators remains to be evaluated.

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