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Peroxiredoxin IV Is a Secretable Protein with Heparin-Binding Properties under Reduced Conditions[1]

Ayako Okado-Matsumoto, Akio Matsumoto, Junichi Fujii,[2] and Naoyuki Taniguchi[3]

Department of Biochemistry, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565-0871

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Peroxiredoxins (PRxs) play a role in protecting protein free thiol groups against oxidative damage and thioredoxin-dependent peroxidase activity. This report describes the characteristics of the fourth member of the mammalian PRxs, PRx IV. Rat PRx IV produced in Sf21 cells by a baculovirus expression system has two bands with different electrophoretic mobilities, 31 and 27 kDa [Matsumoto *et al.* (1999) *FEBS Lett.* 443, 246–250]. The 27-kDa PRx IV lacks the NH_2-terminal 36 amino acids which correspond to a predicted leader peptide, which is required for secretion from cells. Thus, the 31-kDa form is probably a precursor form, and the 27-kDa form, a secretable form which is enzymatically active. Pulse-chase experiments of PRx IV-transfected COS-1 cells showed that PRx IV is processed within 10 min and released from cells. The secretable form contains both reduced and oxidized forms. The reduced form binds to both a heparin affinity column and human umbilical vein endothelial cells, while the oxidized form does not. The equilibrium dissociation constants, K_D, for heparin and heparan sulfate as judged by surface plasmon resonance experiments were 19 and 870 nM, respectively. The secretable form corresponds to the major bands found in most tissues, as evidenced by immunoblot analysis. Within cells, secretable form was largely localized on the endoplasmic reticulum, as judged by colocalization with calreticulin. Moreover, PRx IV has glutathione-dependent peroxidase activity in addition to thioredoxin-dependent activity. These data indicate that PRx IV is a secretable protein and may exert its protective function against oxidative damage by scavenging reactive oxygen species in the extracellular space.

Key words: heparin binding, peroxiredoxin IV, redox, secretable protein, thioredoxin/glutathione-dependent peroxidase.

A number of proteins play a protective role against oxidative stress in living systems. Superoxide dismutases (SODs), glutathione peroxidases (GPxs), and catalase are all well-known antioxidative enzymes which scavenge reactive oxygen species. In addition, more than 50 genes belonging to the same gene superfamily that function as antioxidants have been identified in a wide variety of organisms ranging from prokaryotes to mammals. Collectively, these proteins are referred to as peroxiredoxins (PRxs) (*1*). The encoded proteins are highly conserved in mammalian species such as human, rat, and mouse (*1–8*), and can be divided into five distinct groups, PRxs I, II, III, IV, and V, based on amino acid sequence similarities (*9*). While PRxs I, II, III, and IV contain two conserved cysteine residues (*9*), PRx V contains only one such residue (*10*). PRxs I, II, and V exist in cytoplasm (*10*, *11*), while PRx III is mitochondrial (*7*, *11*). Among the various biological functions ascribed to these proteins, TRx-dependent peroxidase activity appears to be common to all.

PRx IV, which was originally designated as AOE372, was recently isolated from a human cDNA library by using the yeast two-hybrid assay with human PAG (PRx I) as a bait (*9*). The protein is highly homologous with other members of the PRx gene family, with which it exhibits about 70% identities. It also has a distinctive hydrophobic NH_2-terminal sequence, which appears to be a signal sequence responsible for secretion from cells. It has, however, been reported that PRx IV is localized in the cytoplasm, where PRx I and PRx II are also found. One function of this protein is as a regulatory factor for NF-κB, an oxidative stress-activatable transcriptional factor (*12*). PRx IV prevents H_2O_2-induced activation of NF-κB by reducing H_2O_2 in a TRx-dependent manner. Another, quite different function of the same molecule has been reported based on studies of the recombinant protein (*13*). In this case, the same molecule, referred to as TRANK (thioredoxin peroxidase-related

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[2] Present address: Department of Biochemistry, Yamagata University School of Medicine, 2-2-2 Iidanishi, Yamagata, Yamagata 990-9585.

[3] To whom all correspondence should be addressed. Tel: +81-6-6879-3420, Fax: +81-6-6879-3429, E-mail: proftani@biochem.med.osaka-u.ac.jp

Abbreviations: PRx, peroxiredoxin; GPx, glutathione peroxidase; TRx, thioredoxin; DTT, dithiothreitol; GSH, glutathione; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SOD, superoxide dismutase; HUVEC, human umbilical vein endothelial cells; BSA, bovine serum albumin.

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activator of NF-κB and c-Jun N-terminal kinase) was found to function as a cytokine in the activation of NF-κB and as an inducer of nitric oxide synthase in rat astrocytes by a receptor-mediated mechanism. Thus, the localization and function of this gene product are currently the subject of considerable debate.

We recently reported the isolation of the rat PRx IV cDNA homologue by PCR using degenerated oligonucleotide primers which are specific for the conserved Cys residues in PRx families (*14*). When the PRx IV cDNA was expressed in COS-1 cells, the produced protein was 27 kDa, smaller than the molecular size predicted from the cDNA sequences, and was detected both in culture medium and within cells as a soluble form. This suggests that PRx IV is excreted from cells and functions in the extracellular space. Since some antioxidative enzymes, such as extracellular superoxide dismutase (EC-SOD) (*15*) and plasma GPx (GPx 4) (*16*), exist in a plasma form, the extracellular antioxidant activities would also be expected to play a role in the protection of tissues against oxidative injury.

This report describes the characterization of PRx IV produced in a baculovirus expression system. Based on its heparin-binding properties, it is likely that PRx IV protects cells on the extracellular surface and its function is regulated by the redox state of the surrounding tissues.

EXPERIMENTAL PROCEDURES

Materials—Materials were purchased from the following sources: purified *Escherichia coli* thioredoxin reductase and TRx, Sigma; glutathione reductase and GSH, Boehringer Mannheim; dithiothreitol (DTT) and (*p*-amidinophenyl)-methanesulfonylfluoride hydrochloride, Wako Pure Chemicals; *S*-nitroso-*N*-acetyl-D,L-penicillamine was from Dojindo Laboratories; diamide, heparin and cumene hydroperoxide, Nakalai Tesque; NADPH, Oriental Yeast; EZ-link 5-(biotinamide)pentylamine, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and immobilized protein A on trisacryl, Pierce; Ultrogel AcA34 and Ultrogel AcA54, IBF Biotechnics; TSK heparin-5PW column, Tosoh; CM52, Whatman; ECL kit, Sephadex G-25, and L-[^{35}S]cysteine, Amersham Pharmacia Biotech; Centricon-30 and YM-30 membrane filters, Amicon; type I collagen–coated 2-well coculture slides, Becton Dickinson Labware; Dulbecco's modified Eagle's medium, Dulbecco's modified Eagle's medium lacking methionine and cysteine, and MCDB131, Gibco BRL; sensor chip SA and HBS-EP buffer, BIAcore; heparan sulfate and heparitinase (mixture of I and II), Seikagaku Kogyo; FITC-conjugated swine anti–rabbit IgG and mouse anti–human CD54 antibody, Dako; and Cy5-conjugated donkey anti–mouse IgG and rhodamine-conjugated donkey anti–goat IgG, Chemicon International. Goat anti-calreticulin antibody was a gift from Dr. David H. MacLennan, University of Toronto, Canada. All other reagents were of the highest grade available.

Expression in Sf21 Cells—The culture of *Spodoptera frugiperda* (Sf21) cells and manipulation of baculovirus were performed as described previously (*17*). Full-length cDNAs of PRx I, II, and IV (*14*) were ligated to a baculovirus transfer vector pVL1392 (Invitrogen), then purified by CsCl density gradient ultracentrifugation. Each recombinant plasmid (1 μg) was cotransfected into Sf21 cells with 10 ng of baculovirus DNA (BaculoGold, Pharmingen). The recombinant baculovirus carrying the rat PRx cDNAs was amplified to high titer (about 1×10^7 plaque formation units/ml). At 72 h after infection of the recombinant baculovirus, the conditioned media was collected and used for PRx IV purification.

Expression in COS-1 Cells—Full-length PRx IV cDNA was ligated into a pSVK3 expression vector carrying an SV40 early promoter (Amersham Pharmacia Biotech). CsCl-purified pSVK3-PRx IV cDNA (50 μg) was transfected into COS-1 cells by the electroporation method using a Gene Pulser (Bio Rad) at 220 V and 960 μF in ice-cold HEPES-buffered saline, pH 6.95.

Purification of PRx IV from Conditioned Media of Sf21 Cells—All subsequent procedures were carried out at 4°C. The ammonium sulfate concentration in the conditioned media was adjusted to 35% by the addition of a saturated solution. After 30 min, the sample was centrifuged at 10,000 ×*g* for 30 min. The precipitate was dissolved in 20 mM sodium acetate buffer, pH 6.3, and dialyzed overnight against the same buffer. The dialysate was applied to a CM52 column equilibrated with the same buffer. The pass-through fractions were pooled and concentrated with a YM-30 membrane filter, then dialyzed against 10 mM sodium phosphate buffer, pH 7.4. The sample was loaded on a TSK heparin-5PW column in an FPLC system (Pharmacia) and washed at a flow rate of 1 ml/min. The bound PRx IV was eluted with a linear gradient of NaCl (0–1.5 M) in the same buffer. The bound PRx IV fractions, as judged by 12% SDS-PAGE, were concentrated with a YM-30 membrane filter, then further separated by Ultrogel AcA34 gel-filtration chromatography.

Assay of PRx IV Binding to a Heparin Column—PRx IV was loaded on a TSK heparin-5PW column, in conjunction with an FPLC system (Pharmacia), and washed at a flow rate of 1 ml/min. The pass-through fractions were pooled and oxidized with 0.2 mM diamide for 4 h at 4°C, then rechromatographed on the same column. The bound fractions of PRx IV were reduced by 2.5 mM DTT for 4 h at 4°C, then rechromatographed on the same column.

NH_2-Terminal Sequence Analysis—The purified PRx IV protein was separated by 12% SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and stained with Coomassie Brilliant Blue. The portion of the membrane that contained PRx IV was applied directly to a protein sequencing system (G1005A, Hewlett Packard).

Immunoprecipitation—Subconfluent cells in 100-mm-diameter culture dishes were collected in 1.5 ml microcentrifuge tubes and precipitated by centrifugation at 1,500 ×*g* for 5 min. The precipitate was dissolved in 0.5 ml of a lysis buffer (20 mM HEPES, pH 7.4, 0.4 M NaCl, 3 mM EDTA, 1% Triton X-100, 1 μM (*p*-amidinophenyl)methanesulfonylfluoride hydrochloride), rotated for 20 min, and centrifuged at 12,000 ×*g* for 10 min. The resultant supernatant was assayed for protein concentration and used for further analyses. Rat plasma was fractionated by precipitation with 35% ammonium sulfate, and the precipitate was dissolved in one-tenth of PBS. The conditioned medium (10 ml) and cell lysate (150 μg protein) or 500 μl of rat plasma proteins were then incubated with 1 μg of anti–PRx IV IgG as described (*14*) for 1 h at 4°C. After the addition of 10 μl of immobilized protein A gel, the mixture was rotated overnight at 4°C. The immunoprecipitates were collected by centrifugation, washed three times with the lysis buffer,

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and resuspended in an SDS gel loading buffer (60 mM Tris base, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol).

Immunoblot Analysis—Rat tissues were homogenized with a polytron homogenizer in PBS containing 1 μM (*p*-amidinophenyl)methanesulfonylfluoride hydrochloride, 5 mM EDTA, 1 mM benzamidine, 2 μg/ml aprotinin, 2 μg/ml pepstatin, and 2 μg/ml leupeptin on ice. After centrifugation at 105,000 ×*g* for 1 h at 4°C, the supernatant was used for further analysis. Protein samples were separated by 12% SDS-PAGE, then transferred onto nitrocellulose membranes under semi-dry conditions using Trans-blot (Bio-Rad). The membranes were blocked by incubation with 8% skimmed milk in 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, and 0.2% Tween 20 at 4°C overnight, washed four times with the same buffer for 10 min each, then incubated with the diluted (1:2,000) anti–PRx IV IgG at room temperature for 1 h. After washing, the samples were incubated with horseradish peroxidase–conjugated anti–rabbit IgG diluted to 1:4,000 for 40 min at room temperature. After four washings, the chemiluminescence method was employed to amplify the signal using an ECL kit.

Metabolic Labeling—The PRx IV transfected COS-1 cells were preincubated in Dulbecco's modified Eagle's medium lacking methionine and cysteine, but containing 10% dialyzed fetal bovine serum for 1 h at 37°C. The cells were pulse-labeled for 10 min with L-[^{35}S]cysteine (100 μCi/ml). The chase was performed by replacing the medium with the conventional Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The cell lysates and the medium were immunoprecipitated with anti–PRx IV IgG. The immune complexes were separated on SDS-PAGE, and radioactive bands were detected with BAS-2500 (Fuji Film).

Surface Plasmon Resonance Experiments—Heparin and heparan sulfate (10 mg each) were biotinylated by incubation with 1 mM EZ-link 5-(biotinamide)pentylamine and 0.5 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride in 1 ml of 0.1 mM MES, pH 6.5, for 7 h at room temperature. Biotinylated heparin or biotinylated heparan sulfate was separated from unreacted, excess reagents, and the buffer was then replaced with 10 mM HEPES, pH 7.4, containing 0.3 M NaCl by gel filtration on Sephadex G-25.

Surface plasmon resonance experiments were performed on a BIAcore 2000 apparatus (Pharmacia Biosensor) as described perviously (*18*). Binding experiments were performed at 25°C at a flow rate of 5 μl/min using the HBS-EP running buffer (BIAcore). Biotinylated heparin or biotinylated heparan sulfate was immobilized on a streptavidin-precoated sensor chip SA to reach saturation of the surface. After immobilization of heparin or heparan sulfate, 20 μl of a solution containing 10 mg/ml biotin was injected into the immobilized flow cells to block nonspecific binding on the sensor chip SA. To study the PRx IV binding to heparin or heparan sulfate, 20 μl of PRx IV was injected into the immobilized sensor chip SA for the association phase (contact time: 4 min). The concentration of PRx IV was from 0.1 to 8 μM. The surface was regenerated by injecting 20 μl of 2 M NaCl in 10 mM sodium acetate buffer, pH 4.5, followed by 4 M guanidine-HCl in 10 mM sodium acetate buffer, pH 6.0. The dissociation and association rate constants k_{diss} and k_{ass} were calculated by analysis of sensorgrams using BIAevaluation software 2.1 (Pharmacia Biosensor). The equilibrium dissociation constant K_D was calculated as the ratio of k_{diss}/k_{ass}.

Assay of PRx IV Binding to HUVEC—HUVEC were isolated from human umbilical veins as described previously (*19*). The cells were maintained in MCDB131 supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml streptomycin sulfate, 100 U/ml penicillin G, 10 ng/ml recombinant human basic fibroblast growth factor, 10 μg/ml hydrocortisone, and 0.25 μg/ml amphotericin B in a humid atmosphere of 5% CO_2 at 37°C. The experiments were performed using confluent monolayers in 35 mm-diameter culture dishes as follows. The cells were rinsed three times with PBS and preincubated with or without 20 mU of heparitinase for 2 h. They were incubated with 1 ml of PBS containing 100 μg of PRx IV for 2 h at 4°C. To examine the effect of redox state on binding, PRx was preincubated with 0.2 mM H_2O_2, 0.2 mM diamide, or 2.5 mM DTT overnight, and the unreacted reagents were removed by filtration through Centricon-30. The effects of the reagents on the binding of PRx IV to HUVEC during the incubation time were investigated. The effects of the presence at 10 mg/ml of heparin or heparan sulfate during the incubation with PRx IV were also examined. After the incubation, medium containing unbound PRx IV was removed. In some experiments, the cells which had been incubated with PRx IV were washed three times with PBS, and then further incubated with 0.5 ml of PBS containing 10 mg/ml heparin or heparan sulfate, or 1 M NaCl for 10 min at 4°C. After three washings, the cells were lysed in 1 ml of the lysis buffer and subjected to immunoprecipitation followed by immunoblot analysis with anti-PRx IV antibody as the primary antibody.

Immunofluorescent Detection of PRx IV—HUVEC were grown to confluency on type I collagen–coated 2 well coculture slides. To observe the binding of PRx IV to cell surfaces, HUVEC were incubated with 100 μg/ml PRx IV for 2 h. After washing with PBS, the cells were fixed with 3% paraformaldehyde in PBS for 10 min at 4°C, then blocked for 20 min in PBS containing 1% BSA at room temperature. Cells were then incubated for 2 h at room temperature with polyclonal rabbit anti–PRx IV antibody and monoclonal mouse anti-CD54 antibody in PBS containing 1% BSA. After washing with PBS containing 1% BSA, the immunoreacted primary antibodies were visualized with FITC-conjugated swine anti–rabbit IgG (Ex: 488 nm, Em: 515 nm) and Cy5-conjugated donkey anti–mouse IgG (Ex: 633 nm, Em: 655 nm), respectively, by incubation at room temperature for 1 h. To determine the intracellular localization of PRx IV, HUVEC were permealized with 1% saponin in PBS containing 1% BSA, and reacted with the anti–PRx IV IgG and polyclonal goat anti-calreticulin IgG. Rhoamine-conjugated donkey anti–goat IgG (Ex: 543 nm, Em: 570 nm) was used to visualize the binding of the anti-calreticulin IgG. Cell surfaces were observed by fluorescent microscopy and the intracellular localization of permealized cells by fluorescent confocal microscopy with an LSM410 microscope (Carl Zeiss, Germany).

Assay of TRx-Dependent Peroxidase Activity—TRx-dependent peroxidase activity was determined as described previously (*20*) using a reaction mixture containing 46 nM TRx reductase, 2.2 μM TRx, and 250 μM NADPH. One unit of the TRx-dependent peroxidase activity was defined as the amount required to oxidize 1 μmol of NADPH per min at 37°C. The K_m value of PRx IV for TRx was determined by

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measuring activities with 0.1 to 10 μM TRx.

Assay of GSH-Dependent Peroxidase Activity—GSH-dependent peroxidase activity was determined by the method used for the selenocysteine-containing GSH-dependent peroxidase (*21*). The reaction mixture contained 0.1 U glutathione reductase, 1 mM GSH, and 270 μM NADPH; and one unit was defined as the amount required to oxidize 0.5 μmol of NADPH (corresponding to 1 μmol of GSH) per min per mg protein at 37°C. The K_m value of PRx IV for GSH was determined by measuring activities with 0.1 to 10 mM GSH.

Assay of Cell Viability—HUVEC was incubated for 2 h with various concentrations of H_2O_2 in the absence or presence of 100 μg/ml PRx IV which had been treated with 2.5 mM DTT for 18 h. In summary, 2.5 mM DTT was preincubated with PRx IV overnight, then the unreacted reagents were removed by Centricon-30 filtration. The viability of the cells was determined by the dye-exclusion assay using 0.3% trypanblue (*22*).

RESULTS

Characterization of Recombinant PRx IV Overproduced in Sf21 Cells—Of the PRx family genes, the PRx IV gene encodes distinctive hydrophobic NH_2-terminal sequences, typical of the leader peptide for secretory proteins. We recently found that only the 27-kDa form of PRx IV was detected in both conditioned medium and soluble cytosolic fractions in a COS-1 cell expression system (*14*). We then produced and purified rat PRx IV in large quantity in a baculovirus/insect cell expression system, in order to investigate the processing of PRx IV. When PRx IV was overproduced in Sf21 cells by infecting a recombinant virus carrying a rat PRx IV cDNA, two distinct bands (31 and 27 kDa), which were not present in the noninfected control cells, were observed in both the conditioned medium and the soluble fraction of the cells, as evidenced by immunoblot analysis (Fig. 1B). The anti–rat PRx IV antibody (*14*) reacted with proteins in cells infected with only the PRx IV–carrying virus (Fig. 1A). Thus, the antibody against PRx IV was found to be specific for PRx IV among the PRx family members.

We then chromatographically purified the protein. The purified protein also consisted of two components, having molecular sizes of 31 and 27 kDa (Fig. 1C), both of which were recognized by the antibody. Since the elution profiles of these proteins were similar, their complete separation was difficult. However, some fractions contained only the 27-kDa form. We then carried out amino acid sequence analysis of the NH_2-terminus of the two forms, but were able to determine the sequence of only the 27-kDa form, because the NH_2-terminus of 31-kDa PRx IV is blocked. The resulting sequence was Leu-Gln-Gly-Leu-Glu, suggesting that the NH_2-terminal 36 amino acid residues found in the cDNA-deduced sequence were posttranslationally cleaved to give the 27-kDa form. The cleaved peptide fragment corresponding to the hydrophobic region of the protein and the cleavage site between Ala^{36} and Leu^{37} matched the consensus of the processing site of the secretory proteins. The 4-kDa size difference between the two forms closely corresponded to the molecular mass of the 36 amino acids (calculated to be 4.2 kDa). When the TRx-dependent peroxidase activity of the purified PRx IV was assayed, the fraction containing only the 27-kDa species exhibited twice the activity of an equal mixture of 27-kDa and 31-kDa forms (Fig. 1D), suggesting that the 31-kDa form is enzymatically inactive. It is likely that the 31-kDa band is a precursor form and that the 27-kDa band is a secretable form. In the following experiments, except for the assay of peroxidase activity, a mixture of the 31- and 27-kDa forms

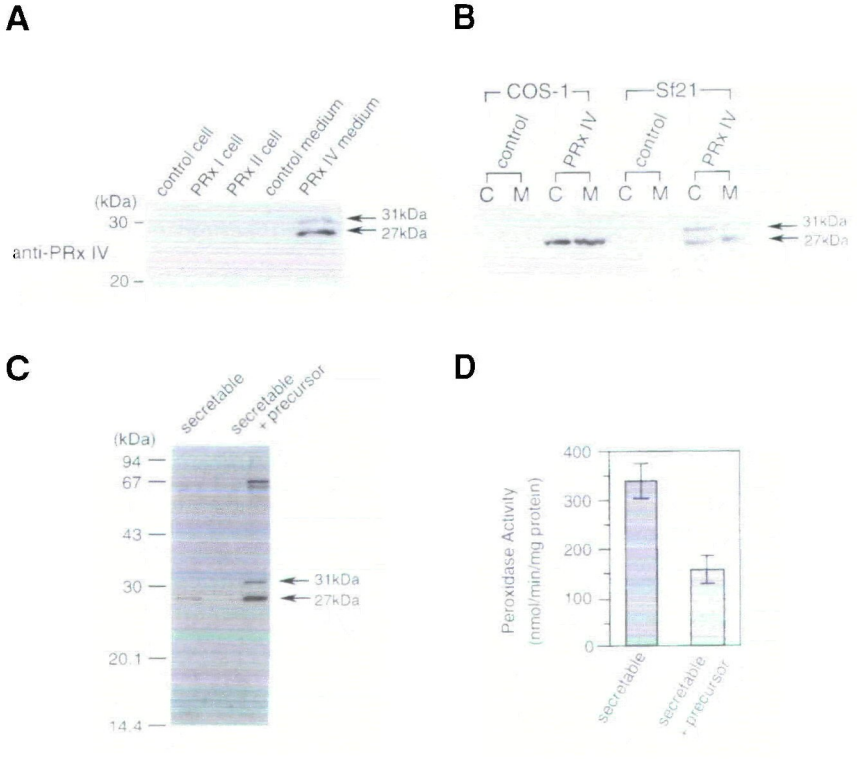

Fig. 1. **Properties of PRx IV produced in COS-1 and Sf21 cells.** A: Specificity of anti-rat PRx IV antibody. Aliquots of 5 μg of proteins from the cytosolic fractions and the conditioned media of Sf21 cells infected with PRx I, II, or IV were analyzed by immunoblotting using the anti-rat PRx IV. B: Aliquots of 5 μg of proteins of the cytosolic fraction (C) and conditioned medium (M) from transfected or nontransfected COS-1 cells and from the infected or noninfected Sf21 cells were analyzed by immunoblotting using the anti–rat PRx IV antibody. C: Secretable and precursor forms of purified PRx IV were separated by 12% SDS-PAGE and visualized by silver staining. D: TRx-dependent peroxidase activities of secretable and precursor forms of PRx IV. Peroxidase activities of PRx IV were examined by measuring the oxidation of NADPH using H_2O_2 as a substrate in the presence of TRx/TRx reductase system. Data are shown as means ± SD of triplicate assays.

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of PRx IV was used.

Metabolic Labeling of PRx IV in the cDNA-Transfected COS-1 Cells—We then performed pulse-chase experiments of PRx IV to clarify the processing of PRx IV. After pulse labeling of COS-1 cells transfected with PRx IV cDNA, in a medium containing [^{35}S]cysteine for 10 min, the reaction was chased with cold complete medium. Immunoprecipitation followed by SDS-PAGE of both cell extracts and medium were then performed for complete samples at each time point. The 27-kDa PRx IV was the only detectable form which was labeled and precipitated with anti–PRx IV IgG from the beginning of this time course (Fig. 2). The amount of labeled PRx IV in the cell gradually decreased during the incubation period. Conversely, labeled PRx IV detected in the medium increased, but the total amounts remained essentially the same. This suggests that most of the PRx IV produced in the cells was immediately processed to the 27-kDa form and was secreted from the cells, like other secretable proteins.

Properties of PRx IV Expressed in Various Rat Tissues and Some Cell Lines—To determine which form of the PRx IV protein was present *in vivo*, the expression of PRx IV in various tissues and cell lines was investigated by immunoblotting (Fig. 3). When soluble fractions extracted from 14 tissues were examined, one positive signal corresponding to 27 kDa was the major form in most tissues and was especially strong in pancreas, liver, and testis. Immunoblots of PRx IV in conditioned media, cytosolic fractions of several cell lines, and rat plasma also showed one major band, 27 kDa in size, corresponding to the major form found in the tissues.

Alterations in Heparin Binding Properties of PRx IV by Redox State—When the mixture of 27-kDa and 30-kDa PRx IV was loaded on a TSK heparin-5PW column in an FPLC system, it was separated into pass-through and bound fractions (Fig. 4A). Both fractions had the same TRx-dependent peroxidase activities (data not shown). Moreover, when the pass-through fractions were treated with 2.5 mM DTT for 4 h at 4°C, they were converted into a form capable of binding to the heparin column (Fig. 4B). However, the bound fractions which had been oxidized with 0.2 mM diamide were no longer able to bind to the column (Fig. 4C). Thus the binding of PRx IV is reversible and regulated by the redox state of the protein. Disulfide formation in the PRx IV molecule may abolish the binding of PRx IV to the heparin column. We further characterized the binding of PRx IV to heparin and heparan sulfate by surface plasmon resonance experiments using a BIAcore 2000 apparatus. The equilibrium dissociation constants K_D for heparin and heparan sulfate were calculated to be 18.6 ± 2.3 nM and 867 ± 143 nM, respectively (Table I). Thus PRx IV exhibited about a 46-fold stronger affinity to heparin than

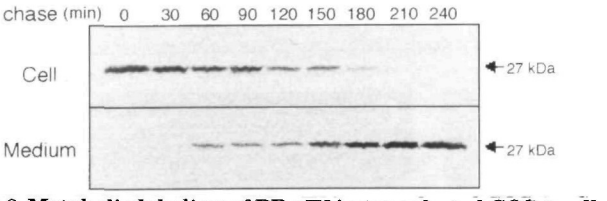

Fig. 2. **Metabolic labeling of PRx IV in transfected COS-1 cells and medium.** PRx IV cDNA–transfected COS-1 cells were pulse-labeled with L-[^{35}S]cysteine for 10 min, then chased with conventional medium. Cells and medium were collected at each time point. The cell lysate and the medium were immunoprecipitated with anti–PRx IV IgG and analyzed by SDS-PAGE. Radioactive bands were detected by BAS2500.

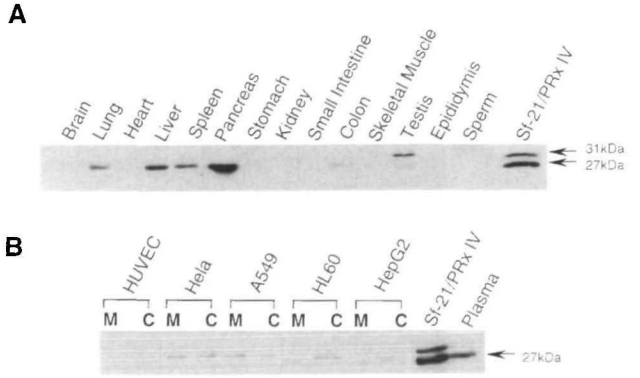

Fig. 3. **PRx IV production in tissues and cell lines.** A: Aliquots of 20 μg of proteins extracted from various rat tissues were analyzed by immunoblot using anti–rat PRx IV antibody. B: Immunoprecipitation of proteins in the cultured medium (M), cellular extracts (C), and rat plasma were carried out with anti–PRx IV antibody. After separation on 12% SDS-PAGE, proteins were reacted with the same antibody, then incubated with peroxidase conjugated–anti rabbit IgG. The chemiluminescence method was employed to amplify the signal using an ECL kit.

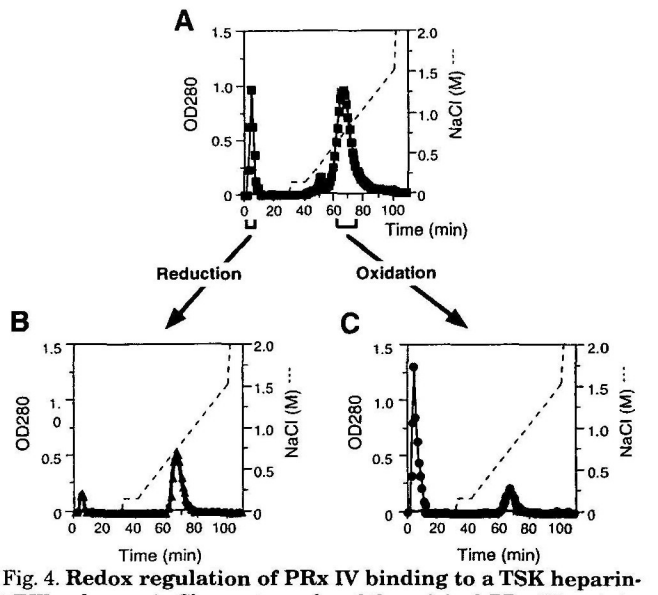

Fig. 4. **Redox regulation of PRx IV binding to a TSK heparin-5PW column.** A: Chromatography of the original PRx IV protein. B: After reduction with 2.5 mM DTT for 4 h at 4°C, the pass-through fractions in A were rechromatographed on the same column. C: After oxidation with 0.2 mM diamide for 4 h at 4°C, the bound fractions in A were rechromatographed on the same column.

TABLE I. **Kinetic binding constants of interactions between PRx IV and immobilized heparin or heparan sulfate.**

	k_{ass} (s^{-1} M^{-1}) ($\times 10^3$)	k_{diss} (s^{-1}) ($\times 10^{-4}$)	K_D (nM)[a]
Heparin	55.7 ± 4.6[b]	1.04 ± 0.12	18.6 ± 2.3
Heparan sulfate	2.0 ± 0.1	1.65 ± 0.15	867 ± 143

[a] $K_D = k_{diss}/k_{ass}$. [b] Data are shown as means ± SD of triplicate assays.

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to heparan sulfate. The equilibrium dissociation constant K_D was similar to that of xanthine oxidase to vascular endothelial cells (K_D = 6 nM) (*23*), and the elution profile of PRx IV was also similar to that of xanthine oxidase from the heparin column (*24*).

Binding Properties of PRx IV to HUVEC in Culture—Since endothelial cells contain heparan sulfate on their cell surfaces, the binding properties of PRx IV to HUVEC were studied (Fig. 5). When HUVEC were incubated with PRx IV at 4°C for 2 h, they were observed to bind PRx IV with moderate affinity. This binding was inhibited by pretreatment with heparitinase or the presence of heparin or heparan sulfate, and the bound PRx IV was released by the addition of these after binding, indicating that PRx IV was bound to heparan sulfate on the cell surface of HUVEC. The treatment of PRx IV with 2.5 mM DTT had no effect on the binding characteristics. However, pretreatment of PRx IV with 0.2 mM diamide or 0.2 mM H_2O_2 abolished the binding capacity to HUVEC. Thus, the binding of PRx IV to HUVEC was *via* heparan sulfate on the cell surface and was also dependent on the redox state of PRx IV. Oxidation of thiol groups of PRx IV by diamide or H_2O_2 might abolish the binding of PRx IV to HUVEC.

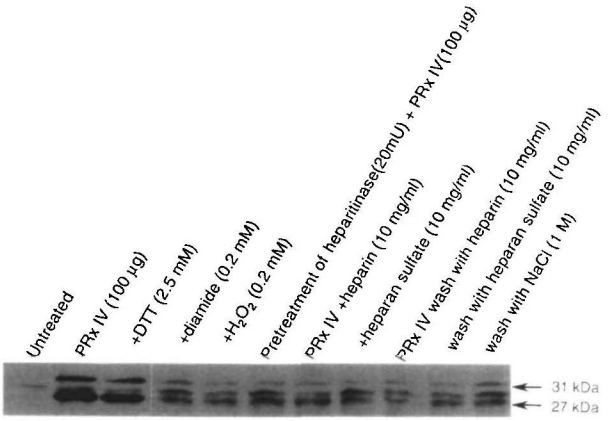

Fig. 5. **Redox regulation of PRx IV binding to HUVEC.** Binding of PRx IV protein to HUVEC surface was shown by immunoblot analysis, which was performed as described in "MATERIALS AND METHODS."

Immunofluorescent Detection of PRx IV—To observe the direct binding of PRx IV to the cell surface of HUVEC, an immunofluorescent study was carried out. After preincubation of intact HUVEC with or without 100 μg/ml PRx IV at 4°C, cells were reacted with rabbit anti-PRx IV IgG and mouse anti-CD54 IgG, then with FITC-labeled anti–rabbit IgG and Cy5-labeled anti–mouse IgG (Fig. 6, a–c). While the control HUVEC, which were preincubated without PRx IV, showed only negligible staining (data not shown), those preincubated with PRx IV exhibited marked fluorescent staining with FITC (Fig. 6a), and the staining profile was similar to that of CD54 (Fig. 6b), a plasma membrane protein, indicating that PRx IV actually bound to the cell sur-

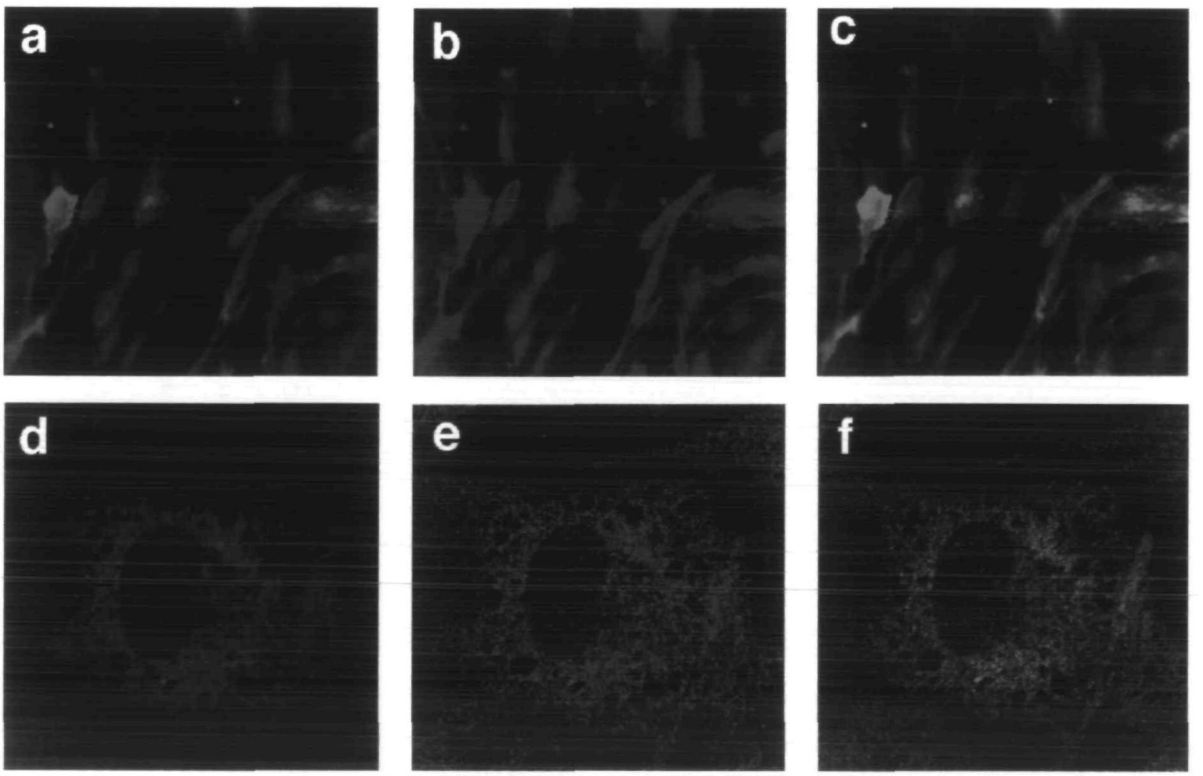

Fig. 6. **Immunofluorescent detection of PRx IV bound to the cell surface and localized at intracellular components.** (a–c) : Intact HUVEC incubated with 100 μg/ml PRx IV were reacted with both rabbit anti–PR IV IgG and mouse anti–CD54 IgG for 2 h, then with FITC-labeled anti–rabbit IgG and Cy5-labeled anti–mouse IgG for 1 h. Fluorescence was detected for FITC (a), Cy5 (b), and FITC + Cy5 (c). (d–f): HUVEC, pretreated with 1% saponin, were reacted with both anti–PRx IV IgG and goat anti–calreticulin IgG for 2 h, then with FITC-labeled anti–rabbit IgG and rhodamine labeled anti–goat IgG for 1 h. Fluorescence was detected for FITC (d), rhodamine (e), and FITC + rhodamine (f).

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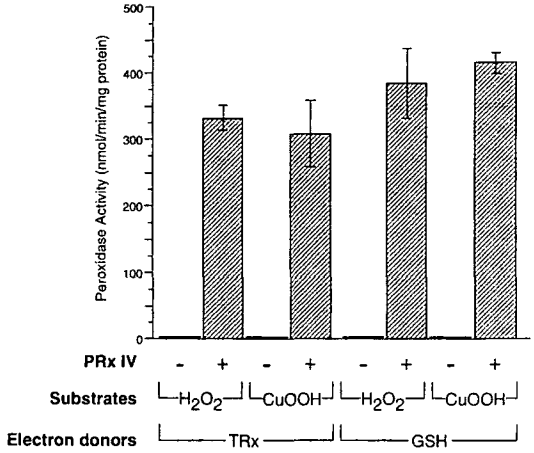

Fig. 7. **TRx- and GSH-dependent peroxidase activities of PRx IV.** Peroxidase activities of PRx IV were examined by measuring oxidation of NADPH using cumene hydroperoxide (CuOOH) or H_2O_2 as substrates in the presence of TRx/TRx reductase and GSH/GSH reductase systems. Data are shown as means ± SD of triplicate assays.

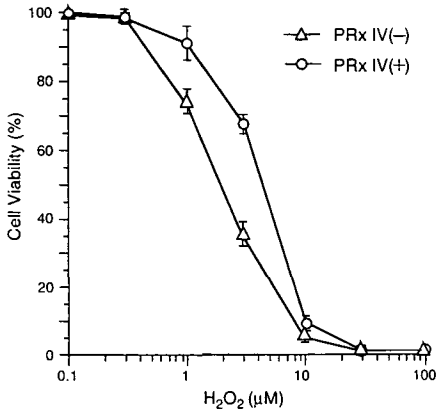

Fig. 8. **Effect of PRx IV on cellular damage caused by H2O2.** The viability of HUVEC in the absence (△) or presence of 100 μg/ml purified DTT-treated PRx IV (○) was determined 18 h after the addition of various concentrations of H_2O_2 to the medium by the dye-exclusion test using 0.3% trypan blue. Data are shown as means ± SD of triplicate assays.

face.

Since some PRx IV existed within cells (Fig. 1), we also investigated the intracellular localization of PRx IV by examining the binding of the anti–PRx IV antibody to cells which had been permealized with saponin (Fig. 6, d–f). The staining profile of the PRx IV with the FITC-labeled second antibody against anti–PRx IV IgG (Fig. 6d) was similar to that of rhodamine-labeled second antibody against anti-calreticulin IgG (Fig. 6e). Since calreticulin is known to be localized on the endoplasmic reticulum and the Golgi apparatus, these data are consistent with PRx IV also being present at these locations. Considering the data in Fig. 2 and the subcellular localization of PRx IV, it can reasonably be concluded that the 27-kDa form which is detected within cells represents the protein in the process of secretion *via* a conventional pathway.

PRx IV Possesses both TRx- and GSH-Dependent Peroxidase Activities—Among the various functions reported for members of the PRx superfamily, TRx-dependent peroxidase activity is the only enzymatic activity hitherto reported that is common to all family members (*11*). Although several proteins which have peroxidase activities are known, selenocysteine-containing GSH peroxidases are major enzymes with high enzymatic activities. We examined whether GSH is capable of donating electrons to PRx IV and of functioning as a cofactor for peroxidase activity. Our results show that PRx IV actually reduced both hydrogen peroxide and cumene hydroperoxide in the presence of a GSH/GSH reductase system as well as the TRx/TRx reductase system (Fig. 7). The specific activities of PRx IV were similar for these electron donors. The K_m values for TRx and GSH were 114 nM and 3.8 mM, respectively. PRx IV of the heparin-bound form and the pass-through form had the same activity. Although selenocysteine-containing GPx was specifically inactivated by a nitric oxide donor, *S*-nitroso-*N*-acetyl-D,L-penicillamine (*25*), neither TRx- nor GSH-dependent peroxidase activity of PRx IV was affected by this reagent.

PRx IV Protects HUVEC against the Cytotoxic Effect of H_2O_2—Since cells which overexpress PRx IV are more resistant to damage caused by H_2O_2, we examined the role of PRx in cellular function in a culture medium. Figure 8 shows the dose-dependent cytotoxicity of H_2O_2 on HUVEC. To avoid direct effects of TRx, TRx reductase, and/or NADPH, a large excess of PRx IV protein alone was used, due to the absence of other reducing equivalents. The presence of 100 μg/ml DTT-treated PRx IV significantly protected the cells against the cytotoxicity of H_2O_2 in the 0.3–10 μM range, a level reached under physiological or pathological conditions (*26*). This supports our conclusion that PRx IV exerts its protective role against peroxides produced in the extracellular space *via* its peroxidase activity.

DISCUSSION

Genes which encode PRxs form a large family of proteins, of which at least five members have been identified in mammals (*9*). The present study demonstrates that PRx IV is synthesized in the form of a 31-kDa precursor with a NH_2-terminal leader peptide containing 36 amino acids, which is then processed to the 27-kDa form. After cleavage of the Ala^{36}-Leu^{37} bond, this is secreted in a manner similar to other secretory proteins. In some cases, however, the signal peptide may remain attached to the polypeptide due to improper processing in the secretory pathway in insect cells using the baculovirus expression vector system (*27*) and the production of very high levels of polypeptide and the shutdown of the host protein synthesis may limit the supply of secretory assistance factors (*28*, *29*). Therefore, in this study, we could detect the 27-kDa form as well as the 31-kDa precursor form using the baculovirus expression vector system. PRx IV is cleaved within 10 min after its synthesis and is released from cells, as evidenced by pulse-chase experiments (Fig. 2). When the TRx-dependent peroxidase activity of purified PRx IV was assayed, the 27-kDa form exhibited twice the activity of an equal mixture of the 31- and 27-kDa forms (Fig. 1D), suggesting that the precursor is inactive. Further studies will be required to clarify the roles of PRx IV forms of different molecular sizes.

The reduced form of PRx IV bound to the heparin column with moderate affinity. Oxidation with diamide con-

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verted PRx IV into a low-heparin-affinity form (Fig. 4). We also examined the binding of PRx IV to HUVEC in culture and found that the binding appears to be regulated by the redox state of the protein itself. Oxidation of PRx IV with diamide or H_2O_2 reduced its binding affinity, causing its release from HUVEC. The complete inhibition of binding by the addition of heparin or heparan sulfate, and the release of the bound PRx IV from cells by washing with heparin, heparan sulfate, or 1 M NaCl suggest that the binding is specific. Thus, it is likely that the binding affinity is reversibly regulated by the redox state of the molecule. Positive charges of clusters containing lysine and arginine residues in EC-SOD (*30*, *31*) and antithrombin III (*32*) are involved in heparin binding. However, in the case of PRx IV, no such cluster exists in the primary structure. A cluster of positive charges may be formed on the surface of the protein when it is folded under reduced conditions. Disulfide formation in PRx IV protein caused by oxidation with diamide or H_2O_2 may induce a conformational change in the molecule and alter the charge distribution involved in heparin binding.

Mammalian PRxs which contain two conserved cysteine residues exhibit peroxidase activity in the presence of the TRx/TRx reductase system (*11*). However, PRxs I, II, and III did not show GSH-dependent H_2O_2-reducing activity. Here, we demonstrate that PRx IV has peroxidase activity in the presence of the GSH/GSH reductase system, as well as the TRx/TRx reductase system. Although a nitric oxide donor, *S*-nitroso-*N*-acetyl-D,L-penicillamine, specifically inactivates selenocysteine-containing GPx activity by oxidizing Sec45 at the catalytic center (*25*), the TRx- and GSH-dependent peroxidase activities of PRx IV were both unaffected by this reagent, probably because of the low reactivity of the essential Cys residue in the catalytic center to NO.

Several peroxidases such as plasma GPx (*33*) and ceruloplasmin (*34–36*) convert hydrogen peroxide to water in plasma. The data herein show that PRx IV is also a peroxide-scavenging enzyme, which functions in the extracellular space. PRx IV binds to the cell surface in a manner similar to EC-SOD, which is attached to the endothelial cell surface *via* glycosaminoglycans (*37*) and is most efficient in the region where oxidative damage primarily occurs. Moreover, PRx IV has the unique characteristic that its localization can be altered by redox state, which may be altered as a result of hypoxia and inflammation. The issue of whether PRx IV binds to the endothelial cell surface may be an important determinant for the prognosis of the damaged tissues. While low molecular weight antioxidants such as glutathione and vitamins A, C, and E are capable of directly interacting with reactive oxygen species, PRx and GPx require an electron from TRx or GSH. Steady-state levels of GSH and TRx in human plasma are reported to be 1.3 μg/ml (*38*) and 37 ng/ml (*39*), respectively. Oxidized GSH and TRx are recycled by reduction with a specific reducing enzyme, *via* the consumption of NADPH. Although it is difficult to assess the physiological significance of the peroxidase activity in plasma at such low concentrations of GSH and TRx, it is established that plasma GPx, whose K_m value for GSH is 4.3 mM (*40*), which is similar to that of PRx IV for GSH (3.8 mM), plays a role in detoxifying peroxides. It is conceivable that PRx IV binds at the inner surface of blood vessels and exerts peroxidase activity, and that severe oxidative stress may release oxidized PRx IV from this location.

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