THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 279, No. 49, Issue of December 3, pp. 50994–51001, 2004
Printed in U.S.A.

Characterization of Mammalian Sulfiredoxin and Its Reactivation of Hyperoxidized Peroxiredoxin through Reduction of Cysteine Sulfinic Acid in the Active Site to Cysteine*

Received for publication, August 18, 2004, and in revised form, September 24, 2004
Published, JBC Papers in Press, September 24, 2004, DOI 10.1074/jbc.M409482200

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Peroxiredoxins (Prxs) are a family of peroxidases that reduce hydroperoxides. The cysteine residue in the active site of certain eukaryotic Prx enzymes undergoes reversible oxidation to sulfinic acid (Cys-SO_2H) during catalysis, and sulfiredoxin (Srx) has been identified as responsible for reversal of the resulting enzyme inactivation in yeast. We have now characterized mammalian orthologs of yeast Srx with an assay based on monitoring of the reduction of sulfinic Prx by immunoblot analysis with antibodies specific for the sulfinic state. Sulfinic reduction by mammalian Srx was found to be a slow process (k_{cat} = 0.18/min) that requires ATP hydrolysis. ATP could be efficiently replaced by GTP, dATP, or dGTP but not by CTP, UTP, dCTP, or dTTP. Both glutathione and thioredoxin are potential physiological electron donors for the Srx reaction, given that their K_m values (1.8 mM and 1.2 μM, respectively) are in the range of their intracellular concentrations, and the V_{max} values obtained with the two reductants were similar. Although its pK_a is relatively low (~7.3), the active site cysteine of Srx remained reduced even when the active site cysteine of most Prx molecules became oxidized. Finally, depletion of human Srx by RNA interference suggested that Srx is largely responsible for reduction of the Cys-SO_2H of Prx in A549 human cells.

Members of the peroxiredoxin (Prx)[1] family of peroxidases catalyze the reduction of H_2O_2 and alkyl hydroperoxides to water and alcohol, respectively, with the use of reducing equivalents provided by thiol-containing proteins (1–4), and they have been detected in organisms from all kingdoms. All of the Prx enzymes contain a conserved cysteine residue in the NH_2-terminal region that is the primary site of oxidation by H_2O_2. The six mammalian Prx isoforms identified to date can be divided into three subgroups that have been designated 2-Cys, atypical 2-Cys, and 1-Cys (2, 5, 6). All of the Prx enzymes exist as homodimers. The 2-Cys proteins, which include Prx I, II, III, and IV, contain an additional conserved cysteine in the COOH-terminal region, whereas members of the atypical 2-Cys (Prx V) and 1-Cys (Prx VI) subgroups do not. The extent of amino acid sequence identity among the three subgroups is low (<20%), whereas that among members of the 2-Cys subgroup is 60–80%.

Among 2-Cys Prx isoforms, Prx I and II are localized in the cytosol, whereas Prx III is restricted to mitochondria, and Prx IV is present in the endoplasmic reticulum as well as in the extracellular space (2). When overexpressed in various cell types, Prx I or Prx II efficiently lowered the intracellular level of H_2O_2 produced in response to cell stimulation with platelet-derived growth factor or tumor necrosis factor, inhibited the activation of nuclear factor-κB induced by tumor necrosis factor, and blocked the induction of apoptosis by ceramide (7, 8), suggesting that Prx enzymes contribute to intracellular signaling by removing H_2O_2.

In the catalytic cycle of 2-Cys Prx proteins, the sulfhydryl group (Cys-SH) of the conserved, H_2O_2-sensitive cysteine residue (Cys^{52} in mammalian Prx I) is selectively oxidized by H_2O_2 to Cys-SOH, which then reacts with the conserved Cys-SH (Cys^{173} in Prx I) located in the COOH terminus of the other subunit in the homodimer to form an intermolecular disulfide. The disulfide is subsequently and specifically reduced by thioredoxin (Trx) (2, 9, 10). Given that the disulfide-forming cysteine residues (Cys^{52} and Cys^{173} in Prx I) of 2-Cys Prx enzymes are situated far apart, with their sulfur atoms separated by ~13 Å (11), formation of the disulfide is a slow process, and the sulfenic intermediate (Cys^{52}-SOH) is occasionally further oxidized to sulfinic acid (Cys^{52}-SO_2H). Trx is not able to reduce this sulfinic acid moiety of Prx, so such hyperoxidation was thought to result in permanent inactivation of peroxidase activity (9, 12, 13). Prokaryotic Prx enzymes, which do not contain the COOH-terminal GGLG motif of their eukaryotic counterparts, are insensitive to oxidative inactivation (14).

Hyperoxidation of 2-Cys Prx enzymes has been detected in various cell types treated with H_2O_2. On examination of the redox state of Prx in several mammalian cell lines that had been metabolically labeled with ^{35}S, however, we previously observed that the intensity of the ^{35}S-labeled acidic spots corresponding to sulfinylated Prx I and Prx II on two-dimensional gels increased during exposure of cells to H_2O_2 and then decreased as the intensity of the spots corresponding to the re-

* This work was supported by the Functional Proteomics Center of the 21st Century Frontier R & D Program and the SK Corporation (to S. M. L. and S. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: Prx, peroxiredoxin; Trx, thioredoxin; Srx, sulfiredoxin; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; AMP-PNP, 5′-adenylyl-β,γ-imidodiphosphate; AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonate; TrxR, thioredoxin reductase; SpTrx, sperm-specific Trx; GST, glutathione *S*-transferase; AEBSF, 4-(2-aminoethyl)benzene-sulfonyl fluoride; siRNA, small interfering RNA.

spective reduced forms of the enzymes increased after removal of H_2O_2 in the presence of the protein synthesis inhibitor cycloheximide (15). This observation led us to propose that the sulfinylation reaction is actually reversible in cells (15, 16).

The enzyme responsible for the reduction of hyperoxidized Prx, sulfiredoxin (Srx), was subsequently identified in yeast on the basis of the observations that its expression was induced by H_2O_2 and that deletion of its gene resulted in a reduced tolerance to H_2O_2 (17). Srx defines a protein family of lower and higher eukaryotes whose members possess a conserved cysteine residue. The fact that Srx is conserved only among eukaryotes is consistent with the observation that prokaryotic Prx enzymes are not susceptible to oxidative inactivation. Studies with the yeast enzyme showed that the reduction of Prx by Srx requires the conserved cysteine of Srx, ATP hydrolysis, Mg^{2+}, and a thiol as an electron donor (17). Sestrins, a family of proteins whose expression is modulated by the tumor suppressor p53, were recently shown to mediate reduction of hyperoxidized Prx in mammalian cells, whereas purified human Srx failed to catalyze such reduction (18). We now show that purified recombinant mammalian Srx enzymes of human, rat, and mouse origin are capable of reducing the Cys-SO_2H moiety of Prx I. In addition, depletion of Srx by RNA interference indicated that Srx is largely responsible for the reduction of Cys-SO_2H of Prx I and II in A549 human lung carcinoma cells.

EXPERIMENTAL PROCEDURES

Materials—Normal human tissue lysates were obtained from DNA Technologies; NADPH, reduced glutathione (GSH), dithiothreitol (DTT), and *N*-ethylmaleimide (NEM) were from Sigma; creatine phosphate and creatine kinase were from USB; $MgCl_2$ was from Alfa Aeser; ATP, ADP, and AMP-PNP were from Calbiochem; sets of ATP, GTP, CTP, and UTP and of dATP, dGTP, dCTP, and dTTP were from Roche Applied Science; and 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonate (AMS) was from Molecular Probes. Human Trx1, rat Trx reductase (TrxR), human Trp14, and recombinant human Prx I were prepared as described previously (19). Recombinant human SpTrx was purified as described (20). Rabbit antiserum to Srx was generated in response to injection either with a purified glutathione *S*-transferase (GST) fusion protein of human Srx or with a keyhole limpet hemocyanin-conjugated peptide (YLGASTPDLQ) corresponding to a sequence in the COOH-terminal region of human, rat, and mouse Srx enzymes. Rabbit antisera specific for the hyperoxidized cysteine-containing Prx enzymes (16), for Prx I (7), or for Trx1 (21) were prepared as described previously. Rabbit antiserum to Trx2 was generated by injection with the purified human Trx2 protein. Monoclonal antibodies to GST and to lamin B were obtained from Santa Cruz Biotechnology and Calbiochem, respectively, and rabbit polyclonal antibodies to protein disulfide isomerase were from Stressgen Biotechnologies. P81 cellulose phosphate paper was from Whatman, and iodo[1-^{14}C]acetamide was from Amersham Biosciences.

Cloning of Mammalian Srx cDNAs—The DNA sequences for human Srx (hSrx), rat Srx (rSrx), and mouse Srx (mSrx) were amplified from IMAGE clone 5735551 (Invitrogen), a rat brain cDNA library (Clontech), and a mouse liver cDNA library (Clontech), respectively, by the polymerase chain reaction. The human Srx cDNA was cloned into the EcoRI-XhoI site of pGEX4T-1 for expression of GST-hSrx or into the NdeI-BamHI site of pET-11a for expression of hSrx alone. The cDNAs for rSrx or mSrx, each containing a sequence encoding a factor Xa-sensitive cleavage site immediately upstream of the initiating methionine codon were subcloned into the EcoRI-XhoI site of pGEX4T-1 for expression of GST-Srx fusion proteins.

Expression and Purification of Recombinant Proteins—Escherichia coli BL21(DE3) or BL21 cells harboring the plasmids encoding hSrx or GST-Srx fusion proteins were cultured at 37 °C in LB medium supplemented with ampicillin (100 μg/ml). After the addition of isopropyl-1-thio-β-D-galactopyranoside (0.1 mM), the cultures were incubated for 3 h at 25 °C, and the cells were then lysed. For purification of hSrx, the cell lysates were prepared by ultrasonic treatment of cells in a solution containing 20 mM HEPES-NaOH (pH 7.0), 1 mM EDTA, and 1 mM 4-(2-aminoethyl)benzene-sulfonyl fluoride (AEBSF). Proteins precipitated by ammonium sulfate (80% (w/v) saturation) were dissolved in 20 mM HEPES-NaOH (pH 7.0) containing 1 mM EDTA and 1 M ammonium sulfate. After removal of debris by centrifugation, the soluble fraction was applied to a TSK Phenyl-5PW column that had been equilibrated with the same solution. The fractions containing hSrx were pooled, concentrated by ultrafiltration (Amicon YM10 device), and dialyzed against 20 mM Tris-HCl (pH 7.4) containing 1 mM EDTA. The dialyzed sample was applied to a Mono-Q HR 5/5 column (Amersham Biosciences) that had been equilibrated with the same solution. The flow-through fraction was collected and dialyzed against 20 mM HEPES-NaOH (pH 7.0) containing 1 mM EDTA. The dialyzed sample was loaded onto a Mono-S HR 5/5 column that had been equilibrated with the same solution, and proteins were eluted with a linear gradient of NaCl from 0 to 0.5 M. The fractions containing hSrx were pooled and dialyzed against 20 mM Tris-HCl (pH 7.4).

GST-Srx proteins were isolated with the use of GSH-Sepharose (Amersham Biosciences) and dialyzed against 20 mM Tris-HCl (pH 7.4). The GST moiety was removed from the fusion proteins by digestion with proteases: factor Xa for rSrx and mSrx and thrombin for hSrx. The resulting digestion mixtures were applied to GSH-Sepharose, and the flow-through fractions were collected. The fractions containing rSrx or mSrx were dialyzed against 20 mM HEPES-NaOH (pH 7.4) containing 1 mM EDTA and then applied to a TSK Heparin-5PW column that had been equilibrated with the same solution. The proteins were eluted with a linear gradient of NaCl from 0 to 0.5 M, and the fractions containing rSrx or mSrx were pooled and dialyzed against phosphate-buffered saline. For purification of hSrx derived from GST-hSrx, the same procedure as that described above for hSrx was followed. The purity and concentration of protein preparations were evaluated by SDS-PAGE and Coomassie Blue staining and by the Bradford assay. The concentration of the recombinant proteins was also determined from measurement of A_{280} and from the extinction coefficient of each protein calculated on the basis of its amino acid composition (22).

Partial Purification of hSrx from A549 Cells—Lysates of A549 cells prepared in a solution containing 20 mM HEPES-NaOH (pH 7.0), 1 mM EDTA, and 1 mM AEBSF were centrifuged, and the resulting supernatant was subjected to ammonium sulfate (80% (w/v) saturation) precipitation. The precipitate was dissolved in a solution containing 20 mM HEPES-NaOH (pH 7.0), 1 mM EDTA, and 0.7 M ammonium sulfate. After removal of debris by centrifugation, the soluble fraction was applied to a TSK Phenyl-5PW column that had been equilibrated with 20 mM HEPES-NaOH (pH 7.0) containing 1 mM EDTA and 1 M ammonium sulfate. The fractions corresponding to the peak of hSrx, detected by immunoblot analysis with antibodies to Srx, were pooled, concentrated by ultrafiltration (Amicon YM10), and dialyzed against phosphate-buffered saline.

Preparation of Sulfinic Prx I—Recombinant human Prx I (2 mg) was incubated in a 1-ml reaction mixture containing 150 μg of Trx1, 1 mM EDTA, 10 mM DTT, 1 mM H_2O_2, and 50 mM Tris-HCl (pH 7.6). Oxidation was initiated by the addition of H_2O_2 and continued for 30 min at 30 °C. The addition of 20 μl of 100 mM H_2O_2 to the reaction mixture and further incubation for 30 min was repeated three times. The reaction mixture was then applied to Q-Sepharose (Amersham Biosciences) that had been equilibrated with 50 mM Tris-HCl (pH 7.6); after incubation for 30 min at room temperature, the resin was separated by centrifugation at 15,000 $\times$ *g* for 1 min. The resulting supernatant was again treated with Q-Sepharose. Sulfinic Prx I free of Trx1 was recovered from the supernatants, and the sulfinic oxidation state of Cys^{52} of the purified protein was confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (13).

Assay of Srx Activity—Reduction of sulfinic Prx I (Ox-Prx I) was initiated by the addition of the indicated concentrations of Srx proteins to a reaction mixture (0.1 ml) containing 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM creatine phosphate, creatine kinase (6.5 units/ml), and various amounts of Ox-Prx I, $MgCl_2$, ATP, and a thiol donor such as GSH or the Trx system. After incubation for the indicated times at 30 °C, portions of the reaction mixture were removed and subjected to immunoblot analysis with antibodies specific for hyperoxidized Prx enzymes (α-Prx-SO_2) and with antibodies to Prx I. The concentration of Ox-Prx I was determined from the intensity of the band corresponding to Ox-Prx I normalized by that of the band for Prx I.

Cell Culture—HeLa (human cervical carcinoma) cells and A549 (human lung carcinoma) cells were maintained in Dulbecco's minimum essential medium and Ham's F-12K medium, respectively, each supplemented with 10% fetal bovine serum and penicillin-streptomycin.

Determination of the Redox State of Srx—A549 cells grown on six-well plates were washed with ice-cold Hanks' balanced salt solutions and then exposed directly to 10% trichloroacetic acid to prevent post-lysis disulfide exchange. The precipitates were washed twice with acetone and suspended in 100 μl of a reaction mixture containing 100 mM

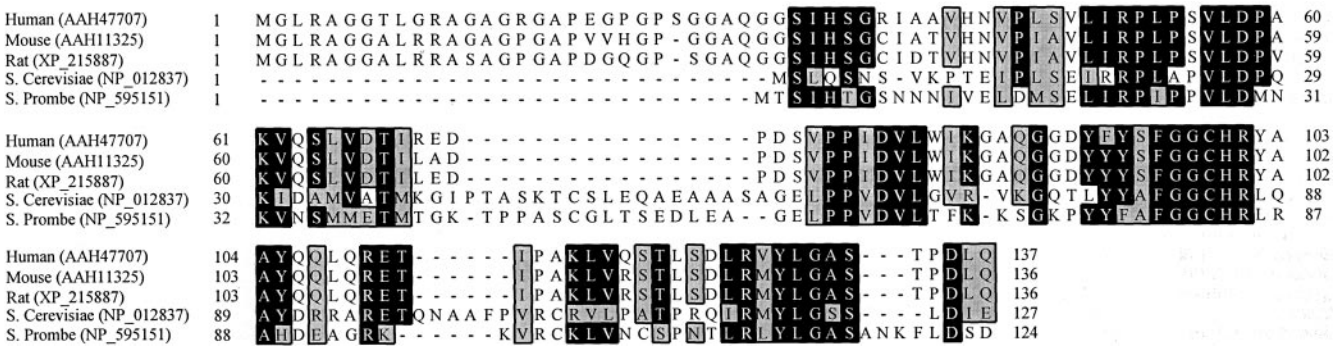

FIG. 1. **Sequence alignment of Srx homologs.** Srx proteins of human, mouse, rat, *S. cerevisiae*, and *S. pombe* were aligned by the ClustalW multiple sequence alignment program. GenBank™ accession numbers are shown in *parentheses*. Identical residues are *boxed* in *black*, and similar residues are *boxed* in *gray*.

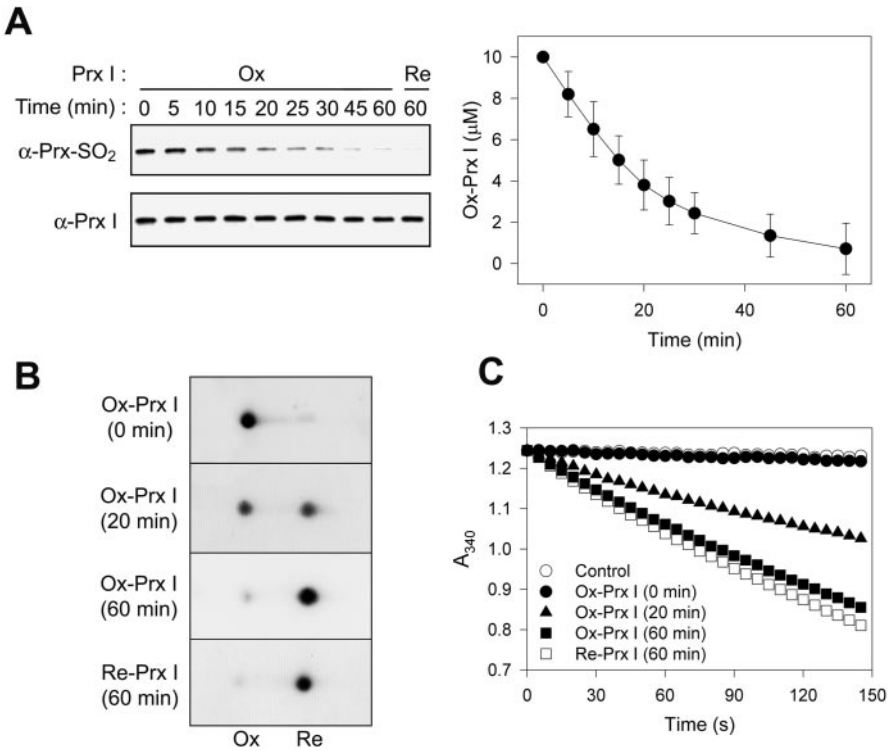

FIG. 2. **Assay of sulfinic reductase activity of Srx with antibodies specific for hyperoxidized Prx.** *A*, a reaction mixture containing 10 μM sulfinic Prx I (Ox-Prx I) or reduced Prx I (Re-Prx I), 1 μM rSrx, 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM creatine phosphate, creatine kinase (6.5 units/ml), 1 mM ATP, 1 mM $MgCl_2$, and 10 mM GSH was incubated at 30 °C for the indicated times and then subjected to immunoblot analysis with antibodies specific for hyperoxidized Prx (α-Prx-SO_2) and for Prx I (α-Prx I) (*left panel*). The concentration of Ox-Prx I remaining in the reaction mixture was determined from the corresponding immunoblot band intensity normalized by the band intensity of Prx I and was then plotted against time (*right panel*); the data are the means ± S.E. from four experiments. *B*, the reaction mixtures containing Ox-Prx I at 0, 20, and 60 min as well as that containing Re-Prx I at 60 min were subjected to two-dimensional PAGE and subsequent immunoblot analysis with antibodies to Prx I. The regions of the immunoblots corresponding to molecular sizes (*vertical*) of 22–28 kDa and to isoelectric points (*horizontal*) of 7.6–8.2 are shown. The positions of oxidized (*Ox*) and reduced (*Re*) Prx I are indicated. *C*, the reaction mixtures containing Ox-Prx I at 0, 20, and 60 min as well as that containing Re-Prx I at 60 min were assayed for peroxidase activity. The oxidation of NADPH coupled by TrxR, Trx1, and Prx I to the reduction of H_2O_2 was monitored at 30 °C as a decrease in A_{340} in a 200-μl reaction mixture containing 50 mM Tris-HCl (pH 7.5), 200 μM NADPH, 50 nM TrxR, 2 μM Trx, 100 μM H_2O_2, and 25 μl of the Srx assay mixture. A reaction mixture lacking Prx I served as a control.

Tris-HCl (pH 8.8), 1 mM EDTA, 1.5% SDS, 1 mM AEBSF, leupeptin (10 μg/ml), aprotinin (10 μg/ml), and either 20 mM NEM or 20 mM AMS. After incubation for 90 min at 30 °C, the reaction was stopped by the addition of nonreducing SDS sample buffer. The samples were resolved by SDS-PAGE on a 14% gel and subjected to immunoblot analysis with antibodies to Srx.

Depletion of hSrx by RNA Interference—A small interfering RNA (siRNA) duplex targeting the 5′-GGAGGUGACUACUUCUACU-3′ sequence in the open reading frame of human Srx mRNA as well as a control RNA duplex of random sequence were obtained from Dharmacon Research. A549 cells (4×10^6) were suspended in 100 μl of solution V (Amaxa Biosystems) with 1 μM siRNA and subjected to transfection by electroporation with program U-17 of the Nucleofector instrument (Amaxa Biosystems).

RESULTS

Preparation of Recombinant Srx of Human, Mouse, and Rat Origin—Whereas yeast Srx was shown to reduce sulfinic Prx in the presence of ATP, Mg^{2+}, and DTT (17), hSrx was found to possess no such sulfinic reductase activity (18). The amino acid sequences of Srx from three mammalian species (human, mouse, and rat) and two yeast species (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) share only 25% identity (Fig. 1). The mammalian enzymes contain ~30 additional residues at their NH_2 termini, whereas the yeast enzymes have ~15 additional residues in their central regions.

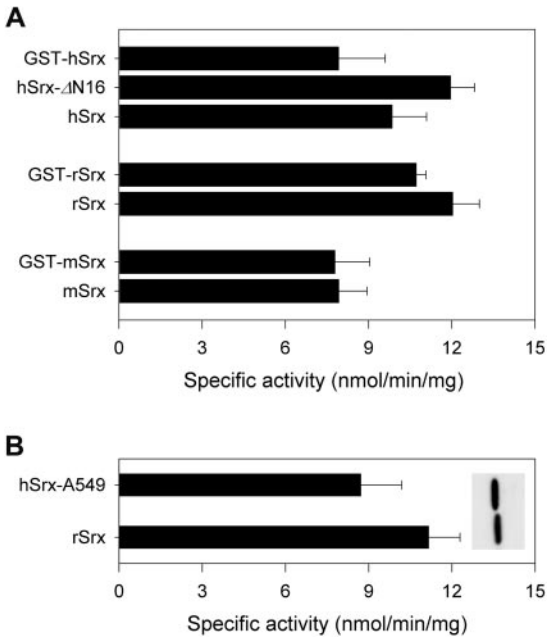

FIG. 3. **Sulfinic reductase activity of various mammalian Srx enzymes.** *A*, the reduction of sulfinic Prx I was performed at 30 °C in a reaction mixture containing 5 μM sulfinic Prx I, 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM creatine phosphate, creatine kinase (6.5 units/ml), 1 mM ATP, 1 mM $MgCl_2$, 10 mM GSH, and 1 μM of the indicated recombinant Srx proteins. The specific activities were calculated from the initial slopes determined by least squares linear regression analysis of the data plotted as shown in Fig. 2*A*. The specific activities of GST-Srx fusion proteins are adjusted for the mass of the GST moiety to allow direct comparisons. *B*, the specific activities of hSrx-A549 and rSrx were determined as in *A*. *Inset*, immunoblot analysis of the reaction mixtures containing hSrx-A549 or rSrx with antibodies specific for the conserved COOH-terminal region of mammalian Srx enzymes. All of the quantitative data are the means ± S.E. from four experiments.

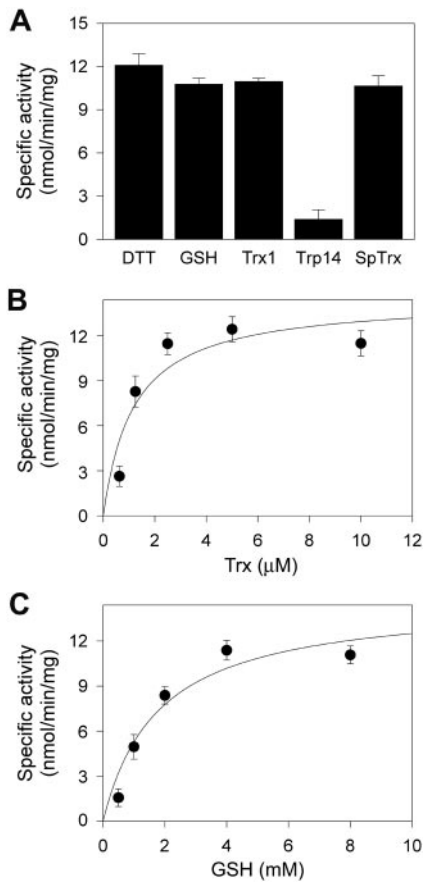

FIG. 4. **Sulfinic reductase activity of rSrx in the presence of various donors of reducing equivalents.** *A*, the reduction of sulfinic Prx I was performed in a reaction mixture containing 10 mM DTT, 10 mM GSH, 5 μM Trx1, 5 μM Trp14, or 5 μM SpTrx as a source of reducing equivalents. For the reaction mixtures containing Trx1, Trp14, or SpTrx, 50 nM TrxR and 200 μM NADPH were also included. The other reaction conditions were as described for Fig. 3*A*. *B* and *C*, initial rates were determined for reaction mixtures containing the indicated concentrations of Trx1 or GSH. The *curves* represent a least squares fit of the Michaelis-Menten equation. The data are the means ± S.E. from four experiments.

To reevaluate the catalytic properties of hSrx, we prepared a GST-hSrx fusion protein that contains a thrombin-sensitive cleavage site between the two moieties. The major product (>90%) of thrombin digestion of GST-hSrx, however, even under conditions of limited proteolysis, was an Srx fragment (hSrx-ΔN16) whose molecular mass determined by mass spectrometry (12777.5 Da) corresponded to that calculated for hSrx lacking the NH_2-terminal 16 amino acids (12776.4 Da). We also prepared recombinant full-length (not fused to GST) hSrx, but it was found to be unstable; freezing-thawing or storage overnight at 4 °C in solutions of various ionic strength or pH resulted in the aggregation of hSrx, whereas hSrx-ΔN16 remained stable under similar conditions (data not shown). We therefore prepared GST fusion proteins of mSrx and rSrx, which generated the respective full-length Srx protein as the main product (>95%) on cleavage with factor Xa (data not shown). The mouse and rat enzymes, either with or without the fused GST moiety, did not show instability on freezing and thawing.

Assay of the Sulfinic Reductase Activity of Mammalian Srx with Antibodies Specific to Hyperoxidized Prx—We previously prepared antibodies to a sulfonylated (SO_3H) peptide modeled on the active site sequence of mammalian 2-Cys Prx enzymes (15). These antibodies (α-Prx-SO_2) recognize sulfinylated and sulfonylated forms of Prx equally well and allow the immunoblot detection of sulfinic Prx I (Ox-Prx I) with high specificity in the presence of excess reduced Prx I (Re-Prx I). We therefore used these antibodies to monitor the reduction of Ox-Prx I (10 μM) by rSrx (1 μM) in the presence of ATP, an ATP-regenerating system (creatine phosphate, creatine kinase), Mg^{2+}, and GSH. The immunoblot intensity of the band detected by α-Prx-SO_2 gradually decreased with time of incubation with rSrx, whereas the intensity of the band detected with antibodies to Prx I remained unchanged (Fig. 2*A*). The rate of reduction of Ox-Prx I by Srx slowed gradually, but it was still possible to determine the initial rate by least squares linear regression analysis (typically with a correlation coefficient of >0.95). We also subjected the reduction reaction mixture both to two-dimensional PAGE (13), which separates the more acidic Ox-Prx I from Re-Prx I (Fig. 2*B*), and to a peroxidase assay, in which Prx-dependent H_2O_2 reduction is coupled to NADPH oxidation in the presence of Trx1 and TrxR (Fig. 2*C*). The results verified that the decrease in immunoblot intensity detected with α-Prx-SO_2 was indeed attributable to the conversion of Ox-Prx I to Re-Prx I and was accompanied by the recovery of peroxidase activity.

With the immunoblot assay, we measured the sulfinic reductase activity of GST-hSrx, hSrx-ΔN16, hSrx (immediately after purification), GST-rSrx, rSrx, GST-mSrx, and mSrx. The specific activities calculated from the initial rates were similar for all these mammalian recombinant enzymes (Fig. 3*A*), suggesting that neither attachment of GST nor deletion of 16 amino acids from the NH_2 terminus had a substantial effect. The range of specific activities, 7–13 nmol/min/mg of protein, is equivalent to a turnover rate (k_{cat}) of 0.1–0.18/min, indicating that the recombinant Srx proteins are highly inefficient enzymes. The reaction rate was also equally slow when measured

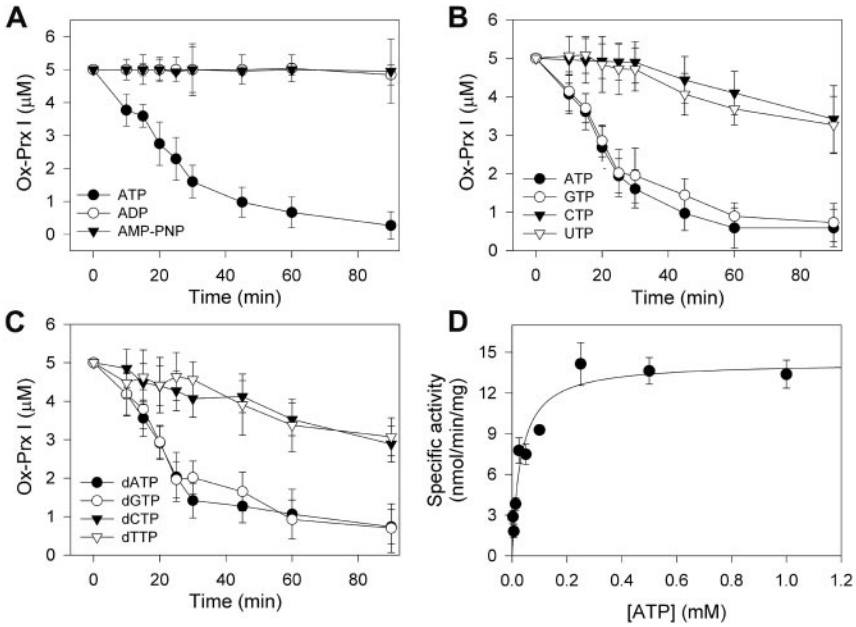

FIG. 5. **Nucleotide dependence of sulfinic reductase activity of rSrx.** *A–C*, the reduction of sulfinic Prx I was performed in a reaction mixture containing the indicated nucleotides at a concentration of 1 mM. The other reaction conditions were as described for Fig. 3*A*. *D*, initial rates of the reaction were determined in the presence of the indicated concentrations of ATP. The data are the means ± S.E. from four experiments.

with sulfinic Prx II as the substrate (data not shown).

It was possible that the low enzymatic activity of the mammalian Srx proteins was an artifact of their recombinant nature. To examine this possibility, we partially purified Srx with a single hydrophobic chromatography step from an extract of human A549 cells. We did not purify the protein further because, like recombinant hSrx, it appeared to be unstable during storage. The partially purified protein (hSrx-A549) was assayed for Srx activity with the immunoblot assay. The amount of hSrx-A549 in the partially purified fraction was estimated from the immunoblot intensity obtained with antibodies to a peptide corresponding to the conserved COOH-terminal region of mammalian Srx enzymes. The specific activity of hSrx-A549 was found to be similar to that of rSrx (Fig. 3*B*), supporting the notion that mammalian Srx is truly an inefficient enzyme.

Physiological Thiol Required for Sulfinic Reductase Activity of Srx—The reductase activity of yeast Srx requires a thiol electron donor such as DTT or Trx (17). We measured the activity of rSrx in the presence of several thiol donors including DTT, GSH, Trx1, a 14-kDa Trx-related protein (Trp14), and SpTrx. Trp14 contains a WC*XX*C motif like that of Trx but shares only 20% amino acid sequence identity with Trx (19), and SpTrx is a novel member of the Trx family of proteins that is expressed exclusively in spermatozoa and consists of a long NH_2-terminal sequence of unknown function and a COOH-terminal sequence typical of thioredoxins (20). With the exception of Trp14, all of these thiol donors exhibited similar activity with rSrx (Fig. 4*A*). The Srx reaction is predicted to generate a sulfinic phosphoryl ester (Cys-SO_2-PO_3^{2-}) intermediate as a result of phosphorylation of Cys-SO_2H (17). Although the active site cysteine of Trp14 is sufficiently nucleophilic and its redox potential is similar to those of other cellular thiol reductants, Trp14 likely is not able to support the Srx reaction probably because the negatively charged phosphorylated intermediate cannot approach the active site surface of Trp14, which appears to be highly negatively charged on the basis of the x-ray crystal structure (24).

To compare the efficacy of GSH and Trx, we analyzed the steady-state kinetics of the reactions supported by these two most abundant cellular thiol donors (Fig. 4, *B* and *C*). The V_{max} values were identical (specific activity, 13 nmol/min/mg), and the K_m values for Trx1 and GSH were 1.2 μM and 1.8 mM, respectively. Given that the intracellular concentrations in mammalian cells are in the range of 1–10 mM for GSH (25) and 2–12 μM for Trx1 (26), both GSH and Trx have the potential to be physiologically relevant electron donors for the Srx reaction.

Nucleotide Specificity of Srx—We measured the sulfinic reductase activity of rSrx in the presence of various nucleotides. Reduction was supported by ATP but not by ADP or the nonhydrolyzable ATP analog AMP-PNP (Fig. 5*A*). At 1 mM, GTP was as efficient as was ATP (Fig. 5*B*); in contrast, yeast Srx is not able to utilize GTP (17). Compared with ATP and GTP, pyrimidine nucleoside triphosphates (CTP and UTP) were substantially less effective (Fig. 5*B*). Similarly, dATP and dGTP were effective, but dCTP and dTTP were far less so (Fig. 5*C*). The K_m for ATP was determined to be ~30 μM (Fig. 5*D*).

Reduced and Sulfinic Prx I Bind to Srx with Similar Affinities—We examined the effect of Re-Prx I on the Srx reaction. The addition of Re-Prx I at 5, 25, or 50 μM to a reaction mixture containing 5 μM Ox-Prx I resulted in concentration-dependent inhibition of rSrx activity (Fig. 6*A*), suggesting that Re-Prx I might compete with Ox-Prx I for binding to Srx. To examine this possibility, we added 1 μg of GST-rSrx or GST to solutions containing 5 μg of Re-Prx I, 2.5 μg each of Re-Prx I and Ox-Prx I, or 5 μg of Ox-Prx I. Proteins precipitated with GST or GST-rSrx were subjected to immunoblot analysis with α-Prx-SO_2 as well as with antibodies to Prx I and to GST (Fig. 6*B*). Immunoblot analysis with antibodies to Prx I indicated that Prx I, independently of its oxidation state, associated with GST-rSrx but not with GST alone. Immunoblot analysis with α-Prx-SO_2 revealed that the amount of sulfinic Prx I precipitated by GST-rSrx from the 1:1 mixture of Re-Prx I and Ox-Prx I was about one-half that precipitated from the reaction mixture containing only Ox-Prx I, suggesting that Re-Prx I and Ox-Prx I bind to rSrx with similar affinities.

To examine the interaction between Prx and rSrx in the presence of other cellular proteins, we prepared lysates of HeLa cells that were either left untreated (Prx I and II in the reduced form) or treated for 20 min with 200 μM H_2O_2 (Prx I and II in the fully oxidized form). The binding assay with GST-rSrx was then performed with each lysate separately or with a 1:1 mixture. Similar to the results obtained with purified Prx I, GST-rSrx bound to reduced Prx I and II and to sulfinic Prx I and II with similar affinities (Fig. 6*B*).

Redox State of the Active Site Cysteine of Srx in H_2O_2-treated Cells—Srx enzymes from all species contain a conserved cys-

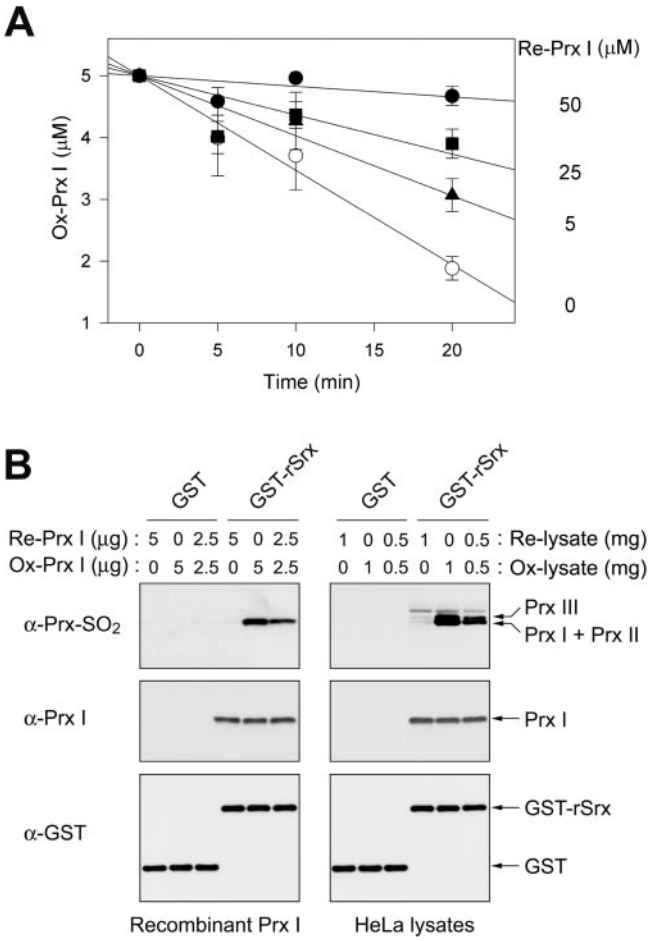

FIG. 6. **Interaction of rSrx with reduced and sulfinic forms of Prx.** *A*, the reduction of sulfinic Prx I (Ox-Prx I, 5 μM) was monitored in the presence of the indicated concentrations of reduced Prx I (Re-Prx I). The other reaction conditions were as described for Fig. 3*A*. The data are the means ± S.E. from three experiments. *B*, the indicated amounts of Re-Prx I and Ox-Prx I were incubated for 2 h at 4 °C with 1 μg of GST or GST-rSrx in 1 ml of a solution containing 50 mM HEPES-NaOH (pH 7.0), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM DTT, 1 mM AEBSF, aprotonin (10 μg/ml), and leupeptin (10 μg/ml). The precipitates obtained with GSH-Sepharose were subjected to immunoblot analysis with the indicated antibodies (*left panels*). In separate experiments, lysates of HeLa cells that were either left untreated (Re-lysate) or treated with 200 μM H_2O_2 for 20 min (Ox-lysate) were prepared; nearly complete hyperoxidation of Prx I and Prx II was confirmed by two-dimensional PAGE (not shown). Binding mixtures containing the indicated amounts of Re-lysate and Ox-lysate were incubated with 1 μg of GST or GST-rSrx, and the resulting precipitates obtained with GSH-Sepharose were subjected to immunoblot analysis as in *A* (*right panels*).

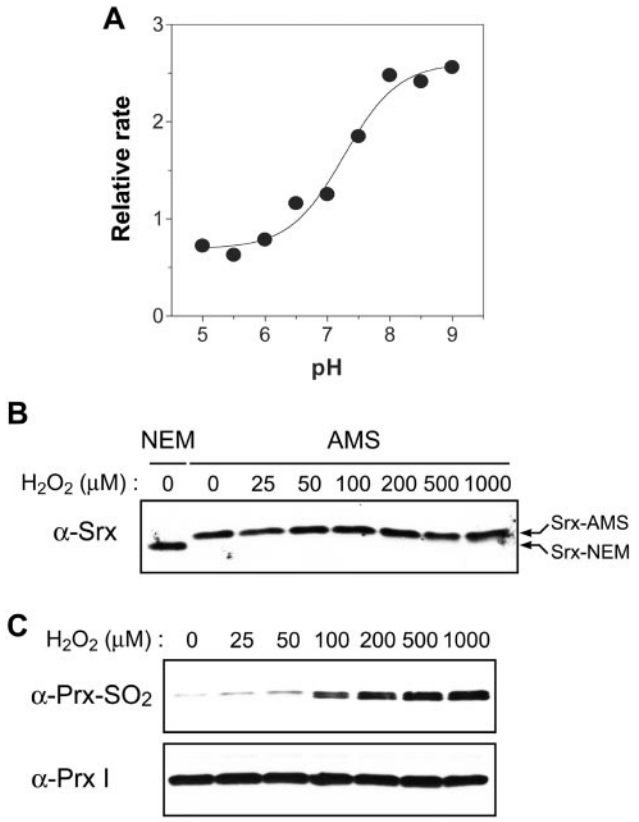

FIG. 7. **Redox properties of the active site cysteine of Srx.** *A*, the pK_a of the active site cysteine of hSrx was determined by the addition of 10 μM hSrx-ΔN16 to a reaction mixture containing 10 μM iodo[1-^{14}C]acetamide (59.0 Ci/mol), 1 mM EDTA, and 100 mM buffer (sodium acetate (pH 5.0), MES-HCl (pH 5.5 to 6.5), HEPES-NaOH (pH 7.0 and 7.5), or Tris-HCl (pH 8.0 to 9.0)). After incubation for 4 min at 30 °C, the reaction was stopped by the addition of 1% phosphoric acid, and samples were spotted onto P81 cellulose phosphate paper. The paper was washed twice with 1% phosphoric acid, rinsed with acetone, and assayed for associated ^{14}C radioactivity. The relative reaction rates derived from the associated radioactivity were plotted against pH. *B*, A549 cells were treated with the indicated concentrations of H_2O_2 for 10 min, after which cell lysates were incubated with NEM or AMS as described under "Experimental Procedures." The samples were then subjected to nonreducing SDS-PAGE followed by immunoblot analysis with antibodies to Srx. *C*, the lysates of H_2O_2-treated A549 cells from *B* were subjected, without thiol modification, to immunoblot analysis with the indicated antibodies.

teine that is essential for catalytic activity (17). Although the role of this cysteine residue in the catalytic mechanism is not yet known, it is thought to serve as the phosphate carrier in the following reaction,

$$\text{Prx-SO}_2\text{H} + \text{ATP} \rightarrow \text{Prx-SO}_2\text{-PO}_3^{2-} + \text{ADP}$$

REACTION 1

or as the phosphate remover in the thiol transferase reaction,

$$\text{Prx-SO}_2\text{-PO}_3^{2-} + \text{Srx-S}^- \rightarrow \text{Prx-S(O)-S-Srx} + \text{PO}_4^{3-}$$

REACTION 2

or as both. Both reactions are facilitated by the thiolate anion (Cys-S$^-$). Most cysteine residues exist in the protonated form (Cys-SH) at neutral pH because their pK_a values (where K_a is the acid constant) are ~8.5. The pK_a of a cysteine residue can be determined from the pH dependence of its reaction with alkylating reagents, which react preferentially with the thiolate anion (27). From the pH dependence of the reaction of hSrx-ΔN16 with [^{14}C]iodoacetamide, the pK_a of the only cysteine residue of hSrx was determined to be ~7.3 (Fig. 7*A*).

Given that thiolate anions react faster with H_2O_2 than do protonated thiols, it was possible that Srx might become oxidized at the active site cysteine and inactivated when its reductase activity is needed to reactivate the sulfinic Prx generated as result of an increased intracellular concentration of H_2O_2. Inactivation of Srx under conditions of oxidative stress would defeat the presumed purpose of its existence, however. To investigate whether the only cysteine residue of hSrx was oxidized in A549 cells that had been exposed to various concentrations of H_2O_2 for 10 min, we subjected cell lysates to alkylation with NEM or AMS. Both AMS and NEM are thiol-reactive reagents that alkylate cysteine residues, thereby adding 500 or 98 Da, respectively, to the molecular mass of each thiol group. The alkylated proteins were fractionated by SDS-PAGE under nonreducing conditions and then subjected to immunoblot analysis with antibodies to Srx (Fig. 7*B*). AMS-

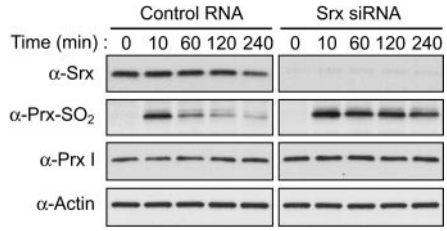

FIG. 8. **Effect of Srx depletion on the reduction of sulfinic Prx in A549 cells.** Thirty-six hours after transfection with a control RNA or hSrx siRNA, A549 cells were exposed to 250 μM H_2O_2 for 10 min, washed with Hanks' balanced salt solution, and incubated for various times in F-12K medium supplemented with 10% fetal bovine serum and cycloheximide (10 μg/ml). The cell lysates (20 μg of protein) were then subjected to immunoblot analysis with the indicated antibodies. Times refer to the total incubation periods, including the initial 10-min exposure to H_2O_2.

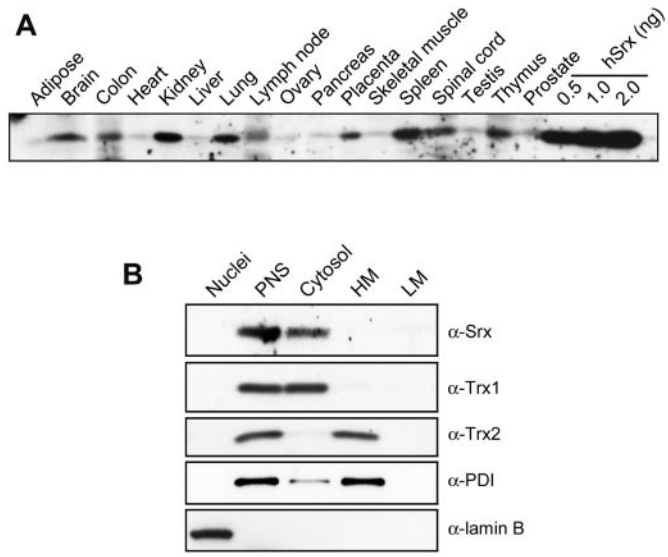

FIG. 9. **Tissue and subcellular distribution of hSrx.** *A*, normal human tissue lysates (20 μg of protein) and the indicated amounts of recombinant hSrx were subjected to immunoblot analysis with antibodies specific for Srx. *B*, nuclear, postnuclear supernatant (*PNS*), cytosolic, and heavy (*HM*) or light (*LM*) membrane fractions (10 μg of protein) prepared from HeLa cells were subjected to immunoblot analysis with the indicated antibodies.

labeled Srx was clearly separated from NEM-labeled Srx, and the intensity of the Srx-AMS band remained unchanged even when the cells were treated with H_2O_2 at concentrations up to 1 mM, suggesting that the active site cysteine of Srx was maintained in the reduced state during exposure of the cells to such high concentrations of H_2O_2. Immunoblot analysis of the same samples with α-Prx-SO_2 indicated that the amounts of sulfinic Prx I and Prx II increased gradually as the concentration of H_2O_2 increased. Srx is able to maintain the thiol state probably not because it is insensitive to oxidation but because its oxidized cysteine is reduced efficiently, similar to the situation with Trx, whose reduction is rapidly catalyzed by TrxR.

Srx Depletion by RNA Interference Retards the Reduction of Sulfinic Prx in A549 Cells—To assess Srx function in cells, we transfected A549 cells with an siRNA specific for hSrx mRNA to deplete Srx selectively. Immunoblot analysis revealed that the amount of Srx was reduced by >95% in cells transfected with the Srx siRNA but was unaffected in those transfected with a control RNA duplex of random sequence (Fig. 8). The transfected cells were then exposed to 250 μM H_2O_2 for 10 min, incubated for various times in H_2O_2-free medium in the presence of cycloheximide, lysed, and subjected to immunoblot analysis. Sulfinic Prx was virtually undetectable in cells transfected with either the hSrx siRNA or the control RNA that had not been exposed to H_2O_2 (Fig. 8). In contrast, α-Prx-SO_2 yielded a pronounced band (sulfinic Prx I and Prx II) in both control RNA- and Srx siRNA-transfected cells that had been incubated with H_2O_2 for 10 min. After removal of H_2O_2, the amount of sulfinic Prx in control RNA-transfected cells decreased much faster than did that in Srx siRNA-transfected cells. The intensity of the bands detected with antibodies to Prx I remained unchanged during the time course. These results thus indicate that Srx depletion retarded the reduction of sulfinic forms of Prx I and Prx II in A549 cells.

Tissue and Subcellular Distribution of hSrx—The expression of hSrx was examined in a variety of human tissues by immunoblot analysis. Only one immunoreactive protein, with a size of ~14 kDa, was detected in all 17 tissues examined (Fig. 9*A*). Comparison of the blot intensities of this protein with those of various amounts of purified hSrx allowed us to estimate the concentration of Srx in several tissues in which it was most abundant, including the kidney, lung, spleen, and thymus; the amounts of Srx in these four tissues were in the range of 5–10 ng/mg of total lysate protein.

To examine the subcellular localization of Srx, we first separated HeLa cell homogenates into nuclear and postnuclear supernatant fractions and then separated the latter into cytosolic, organellar (heavy membrane), and plasma membrane (light membrane) fractions (6). Each fraction was subjected to immunoblot analysis together with the marker proteins Trx1 (cytosol), Trx2 (mitochondria), protein disulfide isomerase (endoplasmic reticulum), and lamin B (nucleus). Although a global analysis of protein localization in budding yeast showed that yeast Srx (Srx1, YKL086W) was present in both cytosol and the nucleus (28) (yeastgfp.ucsf.edu/getOrf.php?orf=YKL086W), hSrx was detected only in the cytosolic fraction of HeLa cells (Fig. 9*B*).

DISCUSSION

Reduction of proteinaceous sulfinic acid in cells was found to occur only recently. Until our demonstration that sulfinic Prx can indeed be reduced in cells, oxidation of cysteine residues to the sulfinic state had been thought to be irreversible. Srx was subsequently identified as the enzyme responsible for the reduction of sulfinic Prx in yeast. The purified recombinant human ortholog of yeast Srx was found not to catalyze the reduction of sulfinic Prx, however. Our results now suggest that this failure to detect the reductase activity of hSrx was likely due to its instability, given that in our hands full-length hSrx readily formed aggregates. In contrast, GST-hSrx and the truncation mutant hSrx-ΔN16 each remained soluble during storage, and recombinant mSrx and rSrx in their full-length forms were also stable.

With the use of immunoblot analysis with antibodies specific for sulfinic Prx and densitometric evaluation of blot intensity, we were able to measure the rate of Srx-dependent reduction of sulfinic Prx I. As shown for yeast Srx, the reduction reaction catalyzed by mammalian Srx requires ATP hydrolysis (it is supported by ATP but not by AMP-PNP or ADP). Unlike yeast Srx, however, mammalian Srx utilized GTP efficiently. Other purine nucleoside triphosphates (dATP and dGTP) also supported the reaction, whereas pyrimidine nucleoside triphosphates (CTP, UTP, dCTP, and dTTP) were less effective. Steady-state kinetic analysis suggested that GSH and Trx provide the reducing equivalents required for the Srx reaction with a similar efficiency; the V_{max} values of the reactions supported by GSH and Trx were identical, and the K_m values were in the range of the corresponding intracellular concentrations.

Kinetic analysis also indicated that the reduction of sulfinic forms of Prx I or Prx II is a slow process, with a turnover rate

of <0.2/min. Given that prokaryotic 2-Cys Prx enzymes are not inactivated by H_2O_2, the oxidative inactivation of eukaryotic 2-Cys Prx isoforms such as Prx I and Prx II has been suggested to be the result of structural features acquired during evolution to accommodate the intracellular messenger function of H_2O_2 (14, 29). Although H_2O_2 has generally been considered a toxic by-product of normal cellular metabolism, much evidence now suggests that the activation of many types of cell surface receptor induces the transient production of H_2O_2, which then functions as an intracellular messenger in the propagation of receptor signaling (23, 30, 31). For this messenger function, it is probably necessary for the concentration of H_2O_2 to increase rapidly above a certain threshold level to induce inactivation of Prx I and Prx II, which are present in large amounts in the cytosol and serve to remove the low levels of H_2O_2 produced as a result of normal cellular metabolism. Hyperoxidation of 2-Cys Prx enzymes has been observed in cells stimulated with tumor necrosis factor (12). We speculate that the slow rate of Prx reactivation via Srx-dependent reduction is also a product of evolution to accommodate the intracellular messenger function of H_2O_2. Rapid reactivation of the inactivated Prx enzymes would reduce the amount of time available for H_2O_2 to accumulate and propagate its signal.

Srx appears to be a ubiquitous enzyme, but its concentration varies greatly among human tissues. The abundance of Srx might thus be an important determinant of how long the H_2O_2 signal persists in a particular cell type. In this regard, it will be of interest to determine whether sestrins, another type of sulfinic reductase, exhibit a high catalytic activity toward sulfinic Prx. In certain cell types, the messenger function of H_2O_2 is not needed, and Prx might exist simply to remove toxic levels of H_2O_2, thus making a rapid reversal of Prx inactivation more desirable.

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Characterization of Mammalian Sulfiredoxin and Its Reactivation of Hyperoxidized Peroxiredoxin through Reduction of Cysteine Sulfinic Acid in the Active Site to Cysteine

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J. Biol. Chem. 2004, 279:50994-51001.
doi: 10.1074/jbc.M409482200 originally published online September 24, 2004
