ANTIOXIDANTS & REDOX SIGNALING
Volume 11, Number 4, 2009

DOI: 10.1089/ars.2008.2360

Original Research Communication

Sestrin 2 Is Not a Reductase for Cysteine Sulfinic Acid of Peroxiredoxins

Hyun Ae Woo, Soo Han Bae, Sunjoo Park, and Sue Goo Rhee

Abstract

The active-site cysteine of 2-Cys peroxiredoxins (Prxs), a subgroup of the Prx family, is reversibly hyperoxidized to cysteine sulfinic acid during catalysis with concomitant loss of peroxidase activity. The reduction of sulfinic 2-Cys Prx enzymes, the first known biologic of such a reaction, has been reported to be catalyzed by either sulfiredoxin (Srx) or sestrin (Sesn) 2. The 13-kDa Srx and 60-kDa Sesn 2 show no sequence similarity, however. Whereas the reductase function of Srx has been confirmed by several studies, such is not the case for Sesn 2. We have now shown that (a) recombinant Sesn 2 did not catalyze the reduction of sulfinic Prx I *in vitro*, whereas Srx did; (b) overexpression of Sesn 2 in HeLa or A549 cells did not affect the reduction of 2-Cys Prxs, whereas overexpression of Srx markedly increased the reduction rate; and (c) the rate of sulfinic 2-Cys Prx reduction in embryonic fibroblasts derived from Sesn 2–knockout mice did not differ from that in those derived from wild-type mice. These results suggest that, unlike Srx, Sesn 2 is not a sulfinic Prx reductase. *Antioxid. Redox Signal.* 11, 739–745.

Introduction

Peroxiredoxins (Prxs) constitute a family of enzymes that catalyze the reduction of hydroperoxides with the use of reducing equivalents provided by a physiologic thiol-containing molecule such as thioredoxin (Trx) (6, 8, 16, 23). Mammalian cells express six isoforms of Prx (Prx I to VI), which are classified into three subgroups (2-Cys, atypical 2-Cys, and 1-Cys) on the basis of the number and position of cysteine residues that participate in catalysis (16, 18). Prx I to Prx IV, which belong to the 2-Cys Prx subgroup, exist as homodimers and possess two conserved cysteine residues. In the catalytic cycle of 2-Cys Prx enzymes, the NH_2-terminal Cys–SH (designated the peroxidatic cysteine, C_P) is first converted to cysteine sulfenic acid (Cys–SOH) by a peroxide. The unstable sulfenic intermediate then reacts with the COOH-terminal conserved Cys–SH (the resolving cysteine, C_R) of the other subunit in the homodimer to form a disulfide, which is subsequently reduced by a thiol-containing reductant, such as Trx, to complete the catalytic cycle (5). As a result of the slow rate of its conversion to a disulfide, the sulfenic intermediate is occasionally oxidized further to cysteine sulfinic acid (Cys–SO_2H) (14, 24). Given that cysteine sulfinic acid is not reduced by biologic reductants such as ascorbic acid, glutathione, or Trx, its formation in 2-Cys Prx isoforms results in the inactivation of peroxidase function.

Sulfinylated Prxs can be separated from their reduced forms by two-dimensional (2D) gel electrophoresis. Studies of the fate of such overoxidized Prx enzymes led to the unexpected finding that the formation of the sulfinic acid form is a reversible step in mammalian cells (20). The enzyme responsible for the reduction of sulfinylated Prx was subsequently identified in yeast and named sulfiredoxin (Srx) (1). Srx defines a protein family of lower and higher eukaryotes whose members possess a conserved cysteine residue. Studies with both yeast and mammalian enzymes have shown that the reduction of sulfinic Prx by Srx requires the conserved cysteine of Srx, ATP hydrolysis, Mg^{2+}, and a thiol as a donor of reducing equivalents (1, 7, 9).

Soon after the discovery of Srx, Budanov *et al.* (2) proposed that members of another family of proteins, designated sestrins (Sesns), possess reductase activity toward cysteine sulfinic acid of Prx. Sesn 1 (PA26) was initially identified as a GADD (growth arrest– and DNA

Division of Life and Pharmaceutical Sciences, Ewha Womans University, Seoul, Korea.

damage–inducible)–related protein whose expression is induced by genotoxic stress in a p53-dependent manner (19), whereas Sesn 2 (Hi95) was identified as a PA26 homologue whose expression is induced in response to prolonged hypoxia or oxidative stress (3). Sestrins were proposed to possess reductase activity on the basis of the observations that (a) depletion of Sesn 1 or Sesn 2 by RNA interference in human embryonic fibroblasts resulted in an increase in the intracellular level of reactive oxygen species; (b) overexpression of Sesn 1 or Sesn 2 potentiated the reduction of the sulfinic form of Prx I in RKO colon cancer cells; and (c) immunopurified FLAG epitope–tagged Sesn 2 catalyzed the reduction of sulfinic Prx I *in vitro*.

Sestrins are 48- to 60-kDa proteins that show no sequence similarity to Srx (13 kDa). The mechanism of the reduction of sulfinic 2-Cys Prx enzymes, the first known biologic example of such a reaction, has been studied extensively by several laboratories (1, 7, 9–11, 13, 17, 21). However, no similar effort has been described for sestrins. To examine how these two different types of protein are able to catalyze the same reaction, we attempted to study the reaction mechanism of Sesn 2. We failed to detect reductase activity of Sesn 2, however, and we therefore conclude that Sesn 2 is not a reductase for cysteine sulfinic acid of Prx.

Materials and Methods

Materials

Preparation of hyperoxidized recombinant human Prx I was described previously (22). Rabbit antisera specific for rat Srx (7), for Prx I (12), or for sulfinic forms of 2-Cys Prxs (22), Prx VI (21), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (21) have also been described. Rabbit antibodies to Sesn 2 and mouse monoclonal antibodies to the hemagglutinin epitope (HA), or to GAPDH, were obtained from Proteintech Group (Chicago, IL), Santa Cruz Biotechnology (Santa Cruz, CA), and Chemicon International (Charlottesville, VA), respectively. Dithiothreitol, ATP, and $MgCl_2$ were obtained from Sigma Aldrich (St. Louis, MO); protein G–Sepharose was from Amersham Biosciences (Piscataway, NJ); H_2O_2 was from Fluka (Milwaukie, WI); and aprotinin, leupeptin, and 4-(2-aminoethyl)-benzenesulfonyl fluoride were from ICN Biomedicals (Costa Mesa, CA). FLAG-tagged Sesn 2 was kindly supplied by Dr. P. Chumakov (Cleveland Clinic Foundation, Cleveland, OH).

Cell culture and transfection

HeLa human cervical cancer cells, A549 human lung epithelial type II cells, and HEK293 human kidney epithelial cells were maintained in Dulbecco's minimum essential medium supplemented with 10% fetal bovine serum and penicillin-streptomycin. Mouse embryonic fibroblasts (MEFs) were prepared at embryonic day 13.5 from embryos obtained by mating Sesn $2^{+/-}$ animals and were maintained in Dulbecco's minimum essential medium supplemented with 10% fetal bovine serum and penicillin-streptomycin. HA-tagged human Srx and Sesn 2 were expressed by cloning of cDNAs into the pCGN vector (21). For expression of nontagged Srx or Sesn 2, the cDNAs were subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA). Cells were transfected with these various vectors with the use of an Amaxa Nucleofector System (Amaxa Biosystems, Koeln, Germany).

Immunoprecipitation and immunoblot analysis

HEK293 cells were transiently transfected with expression vectors for HA-tagged Srx or Sesn 2 or with the corresponding empty vector. Cells were lysed in lysis buffer [50 m*M* Tris-HCl (pH 7.5), 150 m*M* NaCl, 1 m*M* EDTA, 1 m*M* 4-(2-aminoethyl)-benzenesulfonyl fluoride, aprotinin (10 μg/ml), and leupeptin (10 μg/ml)] containing 1% Nonidet P-40, and the cell lysates were centrifuged. The resulting supernatants were subjected to immunoprecipitation with antibodies to HA and protein G–Sepharose, and the immunoprecipitated proteins were assayed for sulfinic reductase activity or were fractionated by SDS–polyacrylamide gel electrophoresis (PAGE) and subjected to immunoblot analysis.

Assay of sulfinic Prx reductase activity

Purified sulfinic Prx I was incubated at 30°C with immunoprecipitated HA-Srx or HA–Sesn 2 in an assay buffer containing 50 m*M* Tris-HCl (pH 7.5), 100 m*M* KCl, 1 m*M* $MgCl_2$, 1 m*M* ATP, and 5 m*M* dithiothreitol. The reaction products collected at the indicated times were subjected to immunoblot analysis with antibodies to sulfinic 2-Cys Prxs. Although the antibodies were actually obtained by immunizing peptides containing a cysteine sulfonic acid, they recognized both sulfinic and sulfonic forms of Prxs (22). Because the active-site sequence (DFTFVCPTEI) is the same for 2-Cys Prxs (Prx I to IV) and because the sizes of Prx I and Prx II are identical, the sulfinic forms of Prx I and Prx II cannot be differentiated by immunoblot analysis. Prx III and Prx IV, however, can be distinguished because their gel electrophoretic mobilities are different.

Sesn 2 knockout mice

The mouse genomic DNA from 129/SvJ mouse J1 embryonic stem cells was screened with PCR by using two-sets of primers to isolate a 2.4-kb *Not*I-*Xho*I fragment derived from the Sesn 2 gene as the 5′-short arm: the forward primer linked *Not*I (5′ gcggccgcagcttaatgcagtca 3′); the reverse primer (5′ tccgctacgatcatggtgtac 3′) and 4.9-kb *Kpn*I-*Kpn*I fragment derived from the *Sestrin2* gene as the 3′-long arm: the forward primer linked *Kpn*I (5′ ggtacccactgaacaactcagg 3′); the reverse primer linked *Kpn*I (5′ ggtacctgaggattaagaccc 3′). We constructed the targeting vector for deleting a segment-contained sequence of region of the Sesn 2 gene to exon1 from exon5 (~11.3 kb), by using a 5′ 2.4-kb short-arm fragment and 3′ 4.9-kb long-arm fragment ligated into the pPNT vector. A targeting vector was designed to replace a ~11.3-kb genomic fragment containing a segment of region of the Sesn 2 gene to exon1 from exon5. A positive selection marker (PGK promoter and neomycin-resistance gene) and a negative selection marker (HSV-1 promoter driven thymidine kinase gene) were appended to the construct to select against nonhomologous recombination. The targeting vector was linearized with *Not*I and electroporated into the 129/SvJ mouse J1 embryonic stem cells.

Clones resistant to G418 and gancyclovir were selected, and homologous recombination was confirmed by Southern blotting. A clone containing the targeted mutation was in-

jected into C57BL/6 blastocysts, and these were subsequently transferred into pseudopregnant foster mothers. The resulting male chimeric mice were bred to C57BL/6 females to obtain heterozygous Sesn 2 mice. Germline transmission of the mutant allele was verified by Southern blot analysis of tail DNA from F_1 offspring with agouti coat color.

All animal experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee.

Results

Sesn 2 does not reduce the cysteine sulfinic acid of Prx I in vitro

We first evaluated the ability of Srx or Sesn 2 to reduce the sulfinic form of Prx I with the use of recombinant HA-Srx or HA–Sesn 2 immunoprecipitated from transiently transfected HEK293 cells with antibodies to HA. The extent of sulfinic Prx I reduction was measured with immunoblot analysis with antibodies that specifically recognize the sequence surrounding the cysteine sulfinic acid at the active site of 2-Cys Prxs (22). The sulfinic form of recombinant Prx I was incubated with HA-Srx, HA–Sesn 2, or a mixture of equimolar amounts of HA-Srx and HA–Sesn 2 in the presence of $MgCl_2$, ATP, and dithiothreitol. The assay mixtures were then fractionated with SDS-PAGE and subjected to immunoblot analysis (Fig. 1A).

To confirm that the amount of HA-tagged proteins included in the reduction mixture was similar, portions of the reaction mixture were immunoblotted with antibodies to HA (Fig. 1B). The intensity of the immunoreactive band obtained with the antibodies to sulfinic 2-Cys Prxs in Fig. 1A gradually decreased during incubation with immunoprecipitated HA-Srx, with the band having almost completely disappeared after 2 h (Fig. 1C). In contrast, the intensity of the immunoreactive band remained largely unchanged after incubation either with a control immunoprecipitate prepared from cells transfected with an empty plasmid or with immunoprecipitated HA–Sesn 2 (Fig. 1C).

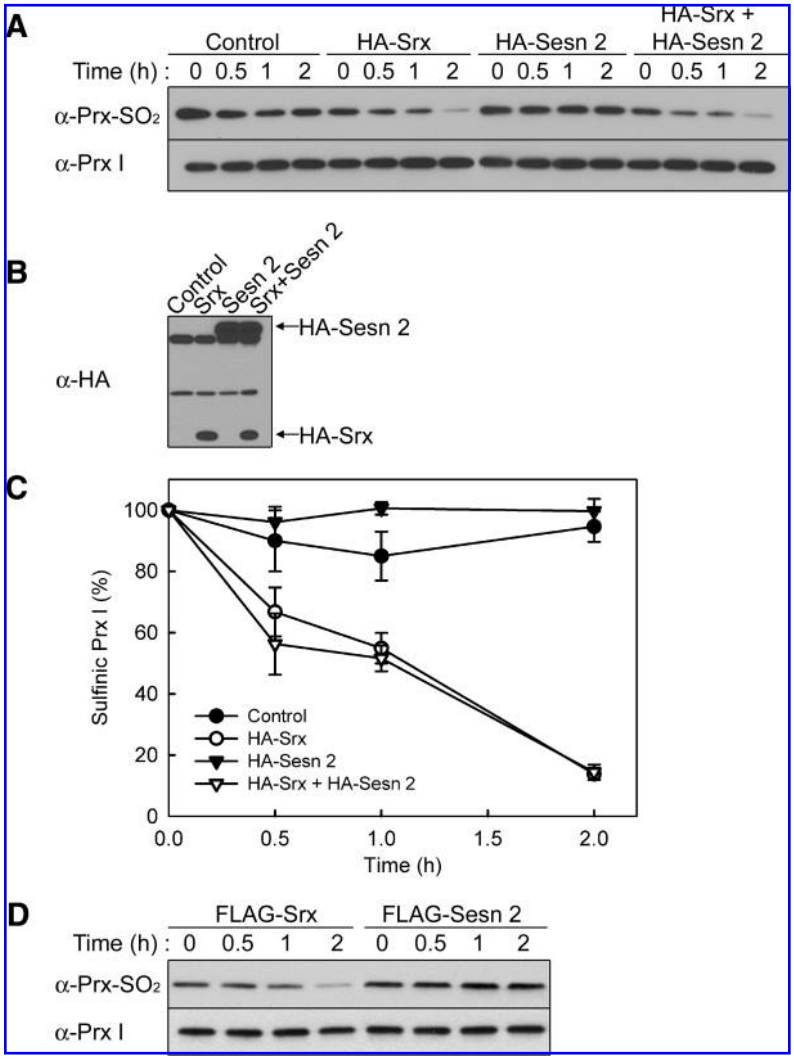

FIG. 1. Sesn 2 does not reduce the cysteine sulfinic acid of Prx I *in vitro*. (**A**) Reduction of sulfinic Prx I by HA-Srx or HA-Sesn 2. Sulfinic human Prx I (1 μM) was incubated at 30°C in a 200-μl reaction mixture containing 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, and 5 mM dithiothreitol. The reaction was initiated by the addition of immunoprecipitated human HA-Srx or HA-Sesn 2, or of both proteins. A control reaction was performed with an immunoprecipitate prepared from cells transfected with an empty vector. Portions (50 μl) of the assay mixture were removed at the indicated times and were subjected to SDS-PAGE and immunoblot analysis with antibodies specific for the sulfinic form of 2-Cys Prxs. The blots were reprobed with antibodies to Prx I. (**B**) Addition of equal amount of HA-Srx and HA-Sesn 2 in **A** was confirmed by immunoblot analysis of those HA-conjugated proteins with antibodies to HA. (**C**) The immunoblot intensities estimated from **A** by a densitometer were plotted against time. Data are expressed as mean ± SEM of values from three independent experiments. (**D**) Reduction of sulfinic Prx I by FLAG-Srx or FLAG-Sesn 2. The reaction condition was identical to that in **A** except that HA-Srx and HA-Sesn 2 were replaced by immunoprecipitated FLAG-Srx and FLAG-Sesn 2, respectively.

The rate of reduction of sulfinic Prx I by immunoprecipitated HA-Srx was not affected by the presence of immunoprecipitated HA–Sesn 2 (Fig. 1C). FLAG-Sesn 2 also did not catalyze the reduction of sulfinic Prx I, whereas FLAG-Srx did (Fig. 1D).

These data thus indicated that, at least *in vitro*, Sesn 2 is neither a reductase for sulfinic Prx I nor an activator of the reduction reaction catalyzed by Srx.

Effect of overexpression of Srx or Sesn 2 on reduction of the cysteine sulfinic acid of 2-Cys Prxs, Prx VI, or GAPDH

We next evaluated the effect of overexpression of Srx or Sesn 2 on the reduction of sulfinic Prx I in HeLa cells (Fig. 2A). Cells that had been transfected with an expression vector for HA-Srx or HA–Sesn 2, or with the corresponding empty vector, were exposed to 250 μM H_2O_2 for 10 min (0.16 h) to induce Prx sulfinylation. They were then incubated for various times in the absence of H_2O_2, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to sulfinic 2-Cys Prxs (Fig. 2B). Given that the active-site sequence (DFTFVCPTEI) is the same for all mammalian 2-Cys Prx proteins (Prx I to IV) and that the sizes of Prx I and Prx II are identical, the sulfinic forms of Prx I and Prx II cannot be differentiated with immunoblot analysis.

We previously showed that HeLa cells contain both Prx I and Prx II and that the sulfinic forms of both Prxs are reduced by Srx (22). HeLa cells also express Prx III, which is slightly larger than Prx I/II and appeared as a faint band slightly above that for Prx I/II in the immunoblot with the antibodies to sulfinic 2-Cys Prxs. The reduction of Prx I/II and Prx III in the cells expressing HA-Srx was much faster than that in the cells transfected with the control plasmid (pCGN). In contrast, expression of HA–Sesn 2 had no effect on the reduction rate.

We previously showed that the active-site cysteine of Prx VI or GAPDH also undergoes hyperoxidation to cysteine sulfinic acid in cells exposed to H_2O_2, but that only the sulfinic acid of 2-Cys Prxs, not that of Prx VI or GAPDH, is reduced by Srx (21). We therefore next tested whether Sesn 2 was able to reduce sulfinic Prx VI. The same HeLa cell lysates used for the analysis of 2-Cys Prxs in Fig. 2B were subjected to immunoblot analysis with antibodies to sulfinic Prx VI. The band for sulfinic Prx VI can be distinguished from those for sulfinic 2-Cys Prxs because the active-site sequence of Prx VI (DFTPVCTTEL) is different from that of 2-Cys Prxs and because specific antibodies are available. The amount of sulfinic Prx VI in cells was not affected by overexpression of Srx or Sesn 2 (Fig. 2C).

We also evaluated the effects of overexpression of Srx or Sesn 2 on reduction of the sulfinic forms of Prx I or Prx III in A549 cells with the use of 2D-PAGE. Lysates derived from cells that had been treated with 1 mM H_2O_2 for 10 min and then incubated in the absence of H_2O_2 for various times were subjected to 2D-PAGE followed by immunoblot analysis. Exposure of the cells to H_2O_2 resulted in the hyperoxidation of almost all Prx I and Prx III molecules, as evidenced by shifts in the corresponding spots to a more acidic position, and these

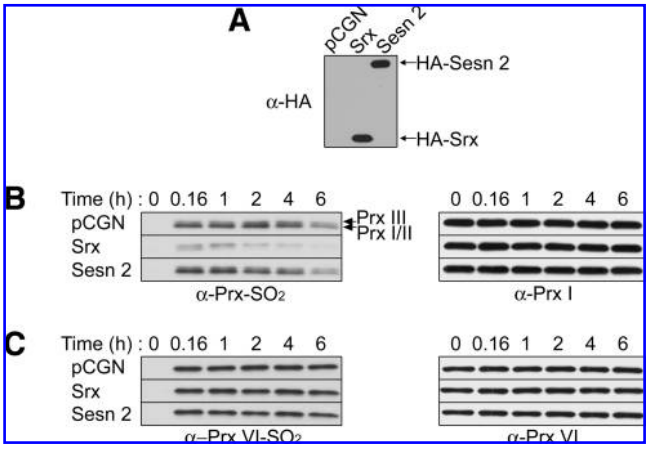

FIG. 2. Lack of effect of Sesn 2 overexpression on the reduction of sulfinic forms of 2-Cys Prxs or Prx VI in HeLa cells. (**A**) Cells were subjected to transient transfection for 36 h with an expression vector for HA-tagged forms of human Srx or Sesn 2, or with the corresponding empty vector (pCGN), after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to HA. (**B**, **C**) The transfected cells were also exposed to 250 μM H_2O_2 for 10 min (0.16 h), washed with phosphate-buffered saline, and incubated for various times in culture medium. Cell lysates were then prepared and subjected to immunoblot analysis with antibodies specific for the sulfinic forms of 2-Cys Prxs (**B**) or of Prx VI (**C**). The blots were reprobed with antibodies to Prx I (**B**) or to Prx VI (**C**). Times shown refer to the total times of incubation, including the 10-min exposure to H_2O_2.

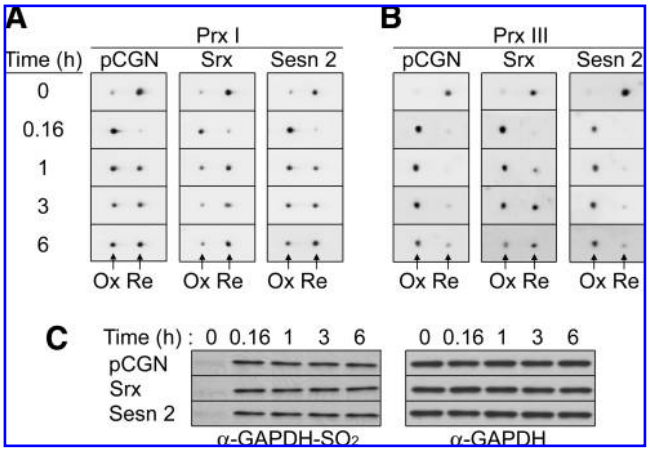

FIG. 3. Lack of effect of Sesn 2 overexpression on the reduction of sulfinic forms of 2-Cys Prxs or GAPDH in A549 cells. Cells subjected to transient transfection for 36 h with an expression vector for HA-tagged forms of human Srx or Sesn 2, or with the corresponding empty vector (pCGN), were exposed to 1 mM H_2O_2 for 10 min (0.16 h), washed with phosphate-buffered saline, and incubated for various times in culture medium. Cell lysates (150 or 20 μg of protein, respectively) were analyzed with 2D-PAGE (**A**, **B**) or SDS-PAGE (**C**), followed by immunoblot analysis with antibodies to Prx I (**A**), to Prx III (**B**), or to sulfinic or total forms of GAPDH (**C**). Times shown refer to the total times of incubation, including the 10-min exposure to H_2O_2. *Ox* and *Re* indicate spots corresponding to the oxidized and reduced forms, respectively, of Prx I or Prx III.

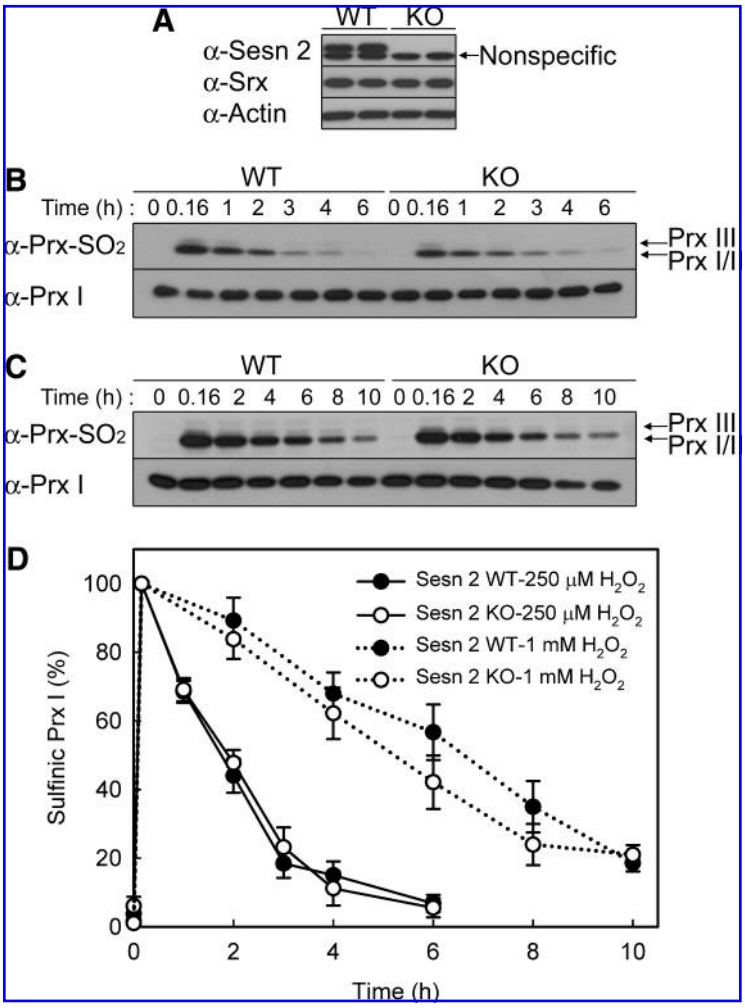

FIG. 4. Lack of effect of Sesn 2 ablation of the reduction of sulfinic 2-Cys Prxs in MEFs. (**A**) Lysates of wild-type (WT) or Sesn 2–knockout (KO) MEFs were subjected to immunoblot analysis with antibodies to Sesn 2, to Srx, or to *β*-actin (loading control). The position of a nonspecific band detected with the antibodies to Sesn 2 is indicated (**B**, **C**). WT or KO MEFs were exposed to 250 *μM* (**B**) or 1 m*M* (**C**) H_2O_2 for 10 min, washed with phosphate-buffered saline, and incubated for various times in culture medium. Cell lysates were then prepared and subjected to immunoblot analysis with antibodies to sulfinic 2-Cys Prxs or to Prx I. Times shown refer to the total times of incubation, including the 10-min exposure to H_2O_2. (**D**) The immunoblot intensities estimated from **B** and **C** by a densitometer were plotted against time. Data are expressed as mean ± SEM of values from three independent experiments.

shifts were gradually reversed after H_2O_2 removal (Fig. 3A and B). The rate of the shift reversal for both Prx I and Prx III spots was greater in cells expressing HA-Srx than in those transfected with the empty plasmid. However, expression of HA–Sesn 2 had no effect on the rate of reduction of either Prx I or Prx III.

The sulfinic form of GAPDH detected with specific antibodies was not reduced in A549 cells expressing either HA-Srx or HA–Sesn 2 (Fig. 3C). Similar experiments performed with A549 cells overexpressing Srx or Sesn 2 without an epitope tag revealed sulfinic reductase activity toward 2-Cys Prxs for Srx but not for Sesn 2 (data not shown).

Lack of effect of Sesn 2 ablation on reduction of the cysteine sulfinic acid of 2-Cys Prxs in MEFs

We previously showed that the reduction of sulfinic 2-Cys Prxs was substantially retarded in cells depleted of Srx by RNA interference (7). To determine the effect of Sesn 2 ablation on this reaction, we prepared MEFs from wild-type or Sesn 2–knockout mice. Our Sesn 2–knockout mice showed no apparent phenotype. Recently, Budanov and Karin (4) published results derived from independently generated sestrin-knockout mice, which also appeared phenotypically normal. Immunoblot analysis confirmed the absence of Sesn 2 in the knockout cells and its lack of effect on the abundance of Srx (Fig. 4A). MEFs were exposed to 250 *μM* or 1 m*M* H_2O_2 for 10 min and then incubated for various times in the absence of H_2O_2, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to sulfinic 2-Cys Prxs (Fig. 4B and C). Regardless of the H_2O_2 concentration used for Prx oxidation, the reduction rate of sulfinic Prx I/II or Prx III was not affected by ablation of Sesn 2 (Fig. 4D).

Discussion

During catalysis of H_2O_2 reduction, the active-site cysteine of Prxs occasionally reacts with two molecules of H_2O_2 and becomes hyperoxidized to cysteine sulfinic acid. Such hyperoxidation results in Prx inactivation. Although Prx is expressed in all organisms examined from prokaryotes to plants and mammals, the prokaryotic enzymes are resistant to hyperoxidation. In addition, prokaryotes do not express Srx. The hyperoxidation of cysteine was thus suggested to represent a mechanism for the regulation of Prx function (15, 23). Although the discovery of Srx and Sesn 2 as reductases for sulfinic Prx provided support for this notion, the lack of any sequence similarity and the disparity in size between Srx and Sesn 2 raised curiosity as to their reaction mechanism. Budanov *et al.* (2) found that Sesn 2, but not Srx, catalyzed the

reduction of sulfinic Prx I *in vitro*. However, several subsequent studies not only confirmed the reductase activity of Srx but also elucidated its reaction mechanism, which involves the specific physical interaction between Srx and 2-Cys Prxs and the formation of a sulfinic phosphoryl ester intermediate (7, 9–11, 13, 17, 21). In contrast, no follow-up reports have appeared on the reductase activity of Sesn 2.

We now provide several lines of evidence that Sesn 2 is not likely a sulfinic reductase. First, we found that recombinant Sesn 2 did not reduce sulfinic Prx I *in vitro*, whereas Srx did. This observation is in direct contrast to that of Budanov *et al.* (2). Second, overexpression of Sesn 2 in HeLa or A549 cells did not affect the reduction of 2-Cys Prxs, whereas that of Srx markedly increased the reduction rate. And last, ablation of Sesn 2 did not affect the reduction rate of 2-Cys Prxs in MEFs. The active-site cysteine of GAPDH or Prx VI also undergoes hyperoxidation, but Srx does not catalyze reduction of the sulfinic forms of these proteins (21). This specificity is due to the fact that Srx physically associates with Prx I to Prx IV but not with other sulfinic proteins. We also found that Sesn 2 did not catalyze reduction of the sulfinic forms of GAPDH or Prx VI.

Acknowledgments

This study was supported by a grant from the Korean Science and Engineering Foundation (National Honor Scientist Program grant 2006-05106) to S.G. R.

Abbreviations

2D, two-dimensional; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, hemagglutinin epitope; MEF, mouse embryonic fibroblast; PAGE, polyacrylamide gel electrophoresis; Prx, peroxiredoxin; Sesn, sestrin; Srx, sulfiredoxin; Trx, thioredoxin.

Disclosure Statement

No competing financial interests exist.

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Address reprint requests to:
Sue Goo Rhee
Division of Life and Pharmaceutical Sciences
Ewha Womans University
11-1 Daehyun-dong, Seodaemun-gu
Seoul 120-750, Korea

E-mail: rheesg@ewha.ac.kr

Date of first submission to ARS Central, November 13, 2008; date of final revised submission, December 8, 2008; date of acceptance, December 29, 2008.

This article has been cited by:

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