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1-Cys peroxiredoxin knock-out mice express mRNA but not protein for a highly related intronless gene

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Abstract 1-Cys peroxiredoxin (1-cysPrx), a member of the peroxiredoxin family with a single conserved cysteine, is a unique antioxidant enzyme. We have generated mice in which the 1-cysPrx gene has been inactivated; they are viable and fertile. Mice have a highly related intronless gene (1-cysPrx-P1, GenBank accession number AF085220) with the same length of open reading frame (224 aa) as 1-cysPrx but located on a different chromosome. Since the product of this gene possibly could mimic 1-cysPrx function, we compared expression of 1-cysPrx and 1-cysPrx-P1 in mouse tissues by real-time polymerase chain reaction and Western blot. 1-cysPrx mRNA and protein were expressed in all mouse tissues that were examined with the highest expression level in lung. 1-cysPrx-P1 mRNA was expressed only in testis. In the 1-cysPrx 'knock-out' mouse, 1-cysPrx-P1 mRNA expression level was similar to the wild type but protein expression was not detected. Thus, mouse 1-cysPrx-P1 is an mRNA-expressed pseudogene that does not result in detectable protein in vivo.

Key words: 1-Cys peroxiredoxin; 1-Cys peroxiredoxin related intronless gene 1; Pseudogene; Knock-out mouse

1. Introduction

1-Cys peroxiredoxin (1-cysPrx) is a unique member of the peroxiredoxin family of antioxidant enzymes that contains a single conserved cysteine [1] and uses glutathione as the physiological reductant [2]. This protein has both glutathione peroxidase and phospholipase A_2 activities [2] and can reduce phospholipid hydroperoxides [3]. Altered expression of 1-cysPrx has been shown in several human diseases including malignant mesothelioma [4] and in a mouse model of altered susceptibility to atherosclerosis [5]. Overexpression of 1-cysPrx can protect cells against phospholipid peroxidation-mediated membrane damage [6] while lipid peroxidation and apoptosis occur when expression is blocked by treatment with an antisense oligonucleotide to 1-cysPrx [7].

Since the 1-cysPrx enzyme appears to play a key role in antioxidant defense, deleting the expression of this gene is expected to result in manifestations of oxidative stress. On the other hand, alternative antioxidant defense pathways might compensate for 1-cysPrx inactivation. A mouse with an inactivated 1-cysPrx gene was generated in order to study this question. A potential limitation to the study of 1-cysPrx function is the recent detection of two intronless murine genes, 1-cysPrx-P1 and 1-cysPrx-P2 (GenBank accession numbers AF085220 and AF085221, respectively), that show high similarity to 1-cysPrx [8,9]. 1-cysPrx-P1 and 1-cysPrx-P2 are located on mouse chromosomes 2 and 4, respectively [9], while 1-cysPrx is located on mouse chromosome 1 [10]. Alignment of the cDNA sequences for these intronless genes with the open reading frame (ORF) sequence of 1-cysPrx revealed ~93% and ~80% identity, respectively [9]. 1-cysPrx-P2 contains a disrupted ORF that would result in a truncated protein that is only 119 amino acids long and would not be expected to exhibit peroxidase activity. On the other hand, 1-cysPrx-P1 shows an intact ORF (224 amino acids) with 86% identity to 1-cysPrx [9]. Inspection of its upstream sequence suggests that it would be differentially regulated as compared to 1-cysPrx [9]. Whether 1-cysPrx-P1 protein is expressed in vivo and its properties are unknown. Of practical importance, expression of 1-cysPrx-P1 protein could complicate studies with 1-cysPrx knock-out mice.

In this study, we inactivated the 1-cysPrx gene in embryonic stem (ES) cells, used the cells to develop mice that were heterozygous for the inactivated gene and then bred them to produce homozygous 1-cysPrx null mice. We used real-time polymerase chain reaction (PCR) to compare the expression levels of 1-cysPrx and 1-cysPrx-P1 in various mouse tissues, studied immunoreactivity of protein expressed by the 1-cysPrx-P1 ORF, and used the 1-cysPrx knock-out mouse to investigate 1-cysPrx-P1 protein expression in vivo. Our study suggests that mouse 1-cysPrx-P1 is an mRNA-expressed pseudogene that does not result in detectable protein in vivo.

2. Materials and methods

2.1. Chemicals and reagents

All primers were synthesized by Qiagen (Valencia, CA, USA). JumpStart™ REDTaq™ DNA polymerase and Deoxynucleotide Mix were obtained from Sigma Chemical Co. (Saint Louis, MO,

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Abbreviations: 1-cysPrx, 1-cys peroxiredoxin; 1-cysPrx-P1, 1-cys peroxiredoxin related intronless gene 1; RT-PCR, reverse transcription-polymerase chain reaction; IPTG, isopropyl-1-thio-β-D-galactopyranoside; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside

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USA). Isopropyl-1-thio-β-D-galactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) were purchased from Fisher Scientific (Pittsburgh, PA, USA). IPTG (100 mM) was prepared in sterile H_2O. X-gal was prepared 20 mg/ml in dimethylformamide. Restriction endonucleases were from New England Biolabs (Beverly, MA, USA). DNA molecular markers, T4 DNA ligase, and Wizard Plus SV Minipreps DNA Purification System were from Promega (Madison, WI, USA). XL10-Gold Ultracompetent Cells were from Stratagene (La Jolla, CA, USA). Shrimp alkaline phosphatase was from Roche (Indianapolis, IN, USA). All other biological reagents, except those noted otherwise, were purchased from Invitrogen (Carlsbad, CA, USA). All chemicals and reagents were used as received.

2.2. Generation of 1-cysPrx knock-out mice and animal care

The previously described BAC genomic clone (clone BACM-153C17 from a 129/SvJ mouse genomic library), which contains the entire functional 1-cysPrx gene [8], was initially obtained from Genome Systems (St. Louis, MO, USA) and used to construct the targeting vector. Briefly, the *Eco*RI genomic fragment containing exons 1 and 2 was cloned into the *Eco*RI site of plasmid pPNT [11], kindly provided by Dr. R. Mulligan of Harvard University, with the orientation of transcription opposite that of the neomycin resistance cassette (neo) and the herpes simplex virus thymidine kinase (TK) gene (Fig. 1A). The *Bam*HI-*Hin*dIII genomic fragment spanning from part of exon 3 to part of exon 5 was initially modified to remove the *Bam*HI restriction site and then inserted into the *Xho*I site of plasmid pPNT. The targeting vector was linearized with the restriction enzyme *Not*I and transfected into R1 mouse ES cells [12], kindly provided by Dr. A. Nagy (Mount Sinai Hospital, Toronto, ON, Canada). The transfected ES cells were selected in medium containing 300 μg/ml of G418 and 2 μM ganciclovir (a gift from Syntex, Palo Alto, CA, USA). Colonies resistant to both drugs were screened by Southern blot analysis using a 0.2 kb *Eco*RI genomic fragment which is external to the genomic sequence in the targeting vector. Using this probe, the signal from the endogenous mouse 1-cysPrx gene was about 6.4 kb and the signal from the targeted allele was about 8.2 kb (Fig. 1A,B). Other smaller bands were detectable as well, presumably originating from the related, intronless copies of 1-cysPrx [8,9]. We screened 471 clones and 16.8% of them were found to contain the targeted 1-cysPrx allele. Clones 126 and 133 were microinjected into C57BL/6J blastocysts according to the method described by Bradley [13], and embryos were re-implanted into the uterine horns of foster mothers. A total of 10 chimeric mice were generated. Male chimeras with more than 95% agouti coat color were chosen to breed with C57BL/6J female mice (Taconic, Germantown, NY, USA). Germline transmission of the ES cell chromosome was achieved with chimeric mice derived from both ES clones. The heterozygous mice containing one inactivated copy of the 1-cysPrx gene were generated at Wayne State University and all procedures were approved by the Animal Investigation Committee.

Offspring that were heterozygous for the mutated 1-cysPrx were then transferred to the University of Pennsylvania for further breeding and characterization. Animal use was reviewed and approved by the University of Pennsylvania Animal Care and Use Committee and mice were cared for in accordance with institutional guidelines. Homozygous 1-cysPrx knock-out mice were generated by intercross of the heterozygous knock-out mice. Screening of offspring for genotype (+/+, +/−, or −/−) was carried out using PCR (Fig. 1C). One reaction amplified the wild type gene; the other amplified the targeted gene. The forward primer for both reactions bound to the 5′ portion of intron 2 that was not deleted in the targeted construct. It had the sequence 5′-GTCTTGATCTGATCCTTCTGTGGAGTCTGC-3′. The reverse primer for detecting the wild type gene bound to the portion of intron 2 that was deleted in the targeting construct. Its sequence was 5′-GGACTCTGAGAAGCAGTTCCTGGATGTTATC-3′. The reverse primer for detecting the targeted gene was located in the neomycin resistance cassette. It had the sequence 5′-CACTGAAGCGGGAAGGGACTGGC-3′. Mouse tissues were removed after anesthesia with intraperitoneal pentobarbital sodium (50 mg/kg) and cervical dislocation, washed with phosphate-buffered saline, snap-frozen in liquid N_2 and stored at −80°C.

2.3. Preparation of genomic DNA

Genomic DNA from mouse tissue was isolated by using the QIAamp DNA Mini Kit (Qiagen). Approximately 1 cm of each mouse tail tip or ∼20 mg mouse liver tissue was used. Briefly, tissues were chopped into small pieces and placed in 180 μl of buffer ATL and digested by 20 μl proteinase K (provided with the kit) at 56°C overnight or until the tissue was completely lysed. After digestion with RNase A, 200 μl buffer AL was added and the solution was incubated at 70°C for 10 min. Then, 200 μl 100% ethanol was added to the sample and the total mixture was applied to a QIAamp spin column. After washing, the genomic DNA was eluted in 200 μl buffer AE and stored at 4°C.

2.4. Isolation of total RNA

Both Trizol Reagent (Life Technologies, Gaithersburg, MD, USA) and RNeasy Mini Kit (Qiagen) were used to isolate total RNA from mouse tissues. Briefly, 50–100 mg of tissue was homogenized in 1 ml of Trizol Reagent using a glass–Teflon and power homogenizer (Talboys Engineering, Emerson, NJ, USA). After incubation for 5 min at room temperature, 0.2 ml of chloroform was added per ml of Trizol Reagent and the solution was mixed vigorously. A colorless upper aqueous phase was separated after centrifugation and total RNA was precipitated by mixing with 0.5 ml of isopropyl alcohol per ml of Trizol Reagent used for the initial homogenization. After washing with 75% ethanol, the RNA pellet was dissolved in 300 μl RNase-free H_2O and then re-extracted with an RNeasy Mini Kit using a slightly modified protocol. RLT buffer (350 μl) was mixed well with 105 μl of the RNA solution. After adding 245 μl of 100% ethanol, the total sample was applied to an RNeasy mini column. On-column DNase digestion was performed by using RNase-free DNase (Qiagen) to remove trace genomic DNA. After washing, total RNA was collected in RNase-free H_2O and its concentration was measured by absorbance at 260 nm with a DU 640B Spectrophotometer (Beckman Coulter, Fullerton, CA, USA).

2.5. RT-PCR and PCR

RT (reverse transcription)-PCR was performed to check the specificity of 1-cysPrx and 1-cysPrx-P1 primers and to detect the mRNA expression of 1-cysPrx and 1-cysPrx-P1 in wild type and 1-cysPrx knock-out mouse tissues. Briefly, 1 μg total RNA was reverse-transcribed into cDNA using a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche). PCR was performed on a Mastercycler (Eppendorf, Westbury, NY, USA) for 33 cycles for both 1-cysPrx and 1-cysPrx-P1. Each cycle included denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and elongation at 72°C for 30 s. The primers for 1-cysPrx and 1-cysPrx-P1 are shown in Fig. 2A. The size of the PCR products was determined by 1.2% or 2.2% agarose gel electrophoresis with visualization by ethidium bromide staining.

The primers used for obtaining the 1-cysPrx-P1 ORF with a His tag were: 5′-ATGCCCGGAGGGTTGCTTCTC-3′ and 5′-TTA$(GTG)_6$-AGGCTGGGGTGTATAACGGAGG-3′. The genomic DNA from mouse liver was used as a template. Pfu Turbo™ DNA Polymerase (Stratagene) was used to generate an accurate blunt-end PCR product. PCR was performed for 40 cycles. Each cycle consisted of denaturation at 95°C for 45 s, annealing at 58°C for 45 s, and elongation at 72°C for 1 min.

2.6. Real-time PCR

To quantify 1-cysPrx and 1-cysPrx-P1 mRNA expression in mouse tissues, real-time PCR was performed on a LightCycler (Roche) using a LightCycler-RNA Master SYBR Green I kit (Roche). Briefly, total RNA was diluted to ∼0.1 μg/μl. The concentration was measured at 260 nm and the sample was diluted again to a concentration of 50 ng/8 μl. Then, 50 ng total RNA from each sample was mixed with 0.3 μM of each primer, 3.0 mM (for 1-cysPrx-P1) or 3.25 mM (for 1-cysPrx and β-actin) $Mn(OAc)_2$, and 1×RNA Master SYBR Green I in a total volume of 20 μl. The experimental protocol consisted of five programs: (1) reverse transcription of template RNA at 61°C for 30 min; (2) denaturation of the cDNA/RNA hybrid at 95°C for 2 min; (3) amplification of cDNA for 45 cycles, each cycle using sequentially 95°C for 5 s, 58°C for 10 s and 72°C for 20 s; (4) analysis of the melting curve to confirm the single product amplification during the PCR assay; (5) cooling the rotor and thermal chamber at 40°C for 30 s. The primers used for real-time PCR are shown in Fig. 2A. The primers for mouse β-actin were: 5′-GGCATTGTTACC-AACTGGGAC-3′ and 5′-ACCAGAGGCATACAGGGACAG-3′. The results were calculated by using the equation: $Ratio = (E_{target})^{\Delta CP_{target}(control-sample)}$ [14]. E_{target} is the real-time PCR efficiency of 1-cysPrx or 1-cysPrx-P1 and ΔCP_{target} is the crossing point (CP)

deviation of control sample of 1-cysPrx or 1-cysPrx-P1. CP is defined as the point at which the fluorescence rises appreciably above background. Standard curves were obtained by real-time PCR using 0.5 ng, 5 ng, and 50 ng total RNA for 1-cysPrx or 1-cysPrx-P1. Real-time PCR efficiencies were calculated from $E = 10^{(-1/\text{slope})}$ using slopes obtained from the standard curves.

2.7. Colony PCR

Colony PCR was performed to select those bacteria containing vectors with the 1-cysPrx-P1 ORF in the correct orientation. White colonies on X-gal/IPTG indicator agar plates were picked using 200 μl sterile pipet tips and transferred to 1.5 ml Eppendorf tubes containing 25 μl of sterile water. Bacterial solution (12 μl) was then transferred to a 200 μl PCR tube, which was placed on a Mastercycler (Eppendorf) at 99°C for 5 min to lyse the cells and denature DNases. After centrifugation to remove cell debris, 5 μl of the supernatant was transferred to a new 200 μl PCR tube for PCR. PCR was performed on a Mastercycler (Eppendorf) for 40 cycles, each cycle using sequentially 95°C for 45 s, 58°C for 45 s, and 72°C for 1 min. The forward primer was in the 1-cysPrx-P1 ORF: 5′-ATGCCCGGAGGGTTGCTTC-TC-3′. The reverse primer was the pETBlueDOWN primer which is in the pETBlue-1 vector: 5′-GTTAAATTGCTAACGCAGTCA-3′. The PCR products were subjected to 1.0% agarose gel electrophoresis and visualized by ethidium bromide staining.

2.8. Construction of 1-cysPrx-P1 expression vector and in vitro translation

The 1-cysPrx-P1 ORF was obtained from mouse liver genomic DNA by PCR as described above and purified from a 1% agarose gel by using QIAquick Gel Extraction Kit (Qiagen). The purified 1-cysPrx-P1 ORF DNA fragment was then inserted into the *Eco*RV enzyme site of a bacterial expression vector, pETBlue-1 (Novagen, Madison, WI, USA) and transformed into *Escherichia coli* XL-10 Gold ultracompetent cells (Stratagene). The positive bacterial clones with correct orientation were first screened by using colony PCR as described above, then confirmed by sequencing using a T7 promoter primer (5′-TAATACGACTCACTATAGGG-3′) and pETBlue-DOWN primer (shown above) in the pETBlue-1 vector. The plasmid DNA containing the correct orientation of the 1-cysPrx-P1 ORF, isolated by using the Wizard *Plus* SV Minipreps DNA Purification System (Promega), was then transformed into Tuner™ (DE3) pLacI *E. coli* expression host cells (Novagen), and a single colony was cultured in Terrific Broth (Life Technologies) containing 1% glucose. When the OD_{600} of the culture was approximately 0.7, 1-cysPrx-P1

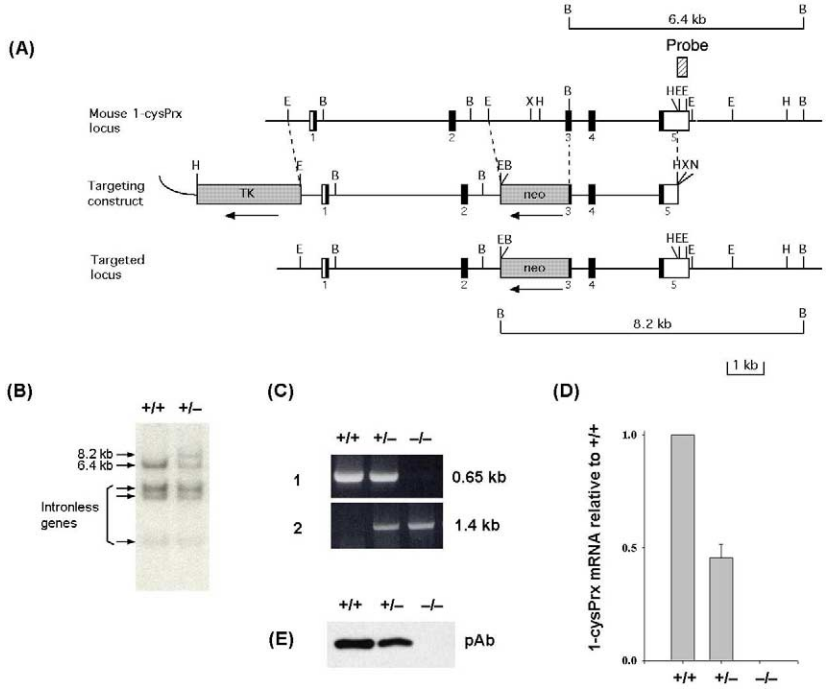

Fig. 1. Targeted disruption of the mouse 1-cysPrx gene. A: Genomic structure and partial restriction map of the mouse 1-cysPrx (*Prdx6*) locus (ENSMUSG00000026701, www.ensembl.org) (top), the targeting vector (middle), and the targeted locus (bottom) are shown. The black boxes represent the protein coding regions in the exons and the open boxes represent the non-coding regions. The exon number is indicated under each exon. The shaded box on top of the restriction map of the 1-cysPrx locus represents the 3′ external sequence used for probing the DNA blot filters. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; N, *Not*I; X, *Xho*I; neo, neomycin resistance cassette; TK, herpes thymidine kinase gene. The neo cassette contained a promoter and polyadenylation site from the mouse phosphoglycerate kinase-1 (PGK-1) gene flanking the neomycin resistance gene. Both neo and TK gene expression were under the control of the mouse PGK-1 promoter located in the neo cassette. The arrow indicates the orientation of transcription of the neo and TK genes. The sizes of the *Bam*HI restriction fragments from wild type and targeted loci hybridized with the probe are shown on the top and bottom of the figure, respectively. B: Southern blot analysis of wild type ES cells and recombinant ES cells carrying mutated 1-cysPrx allele. Genomic DNA isolated from ES cells was digested with *Bam*HI and probed with the 3′ external probe shown in A. +/+ and +/− represent wild type and heterozygous recombinant ES cells, respectively. The 6.4 kb hybridization band is derived from the wild type allele, and the 8.2 kb hybridization band from the mutated allele. C: PCR genotyping of mice. Genomic DNA from mouse tails was screened by PCR as described in Section 2. Panel 1: PCR detection of the intact 1-cysPrx gene was carried out using a forward primer binding to the portion of intron 2 of the mouse 1-cysPrx gene that is not replaced in the targeting strategy and a reverse primer that also binds in intron 2 but to a region that is deleted in the targeted locus. Panel 2: PCR detection of the targeted construct was carried out using the same forward primer as that used for the intact gene while the reverse primer binds in the neomycin resistance cassette that only exists in the targeted locus. In C–E, +/+, +/−, and −/− represent homozygous wild type, heterozygous, and homozygous knock-out mice, respectively. D,E: Expression of 1-cysPrx in mouse lung. D: 1-cysPrx mRNA expression in mouse lung was measured by real-time PCR as described in Section 2. The expression level in the lungs of homozygous wild type mice was set to 1. Results are the mean ± S.E.M. for $n = 3$. E: Levels of 1-cysPrx protein in mouse lung. 1-cysPrx protein (10 μg/lane) from +/+, +/−, and −/− mice was analyzed by Western blot of a reduced gel using the polyclonal anti-peptide antibody to 1-cysPrx.

protein expression was induced by adding 1 mM IPTG to the culture medium. After 3 h, bacteria were collected and soluble protein was extracted with lysis buffer (100 mM NaH_2PO_4, 10 mM Tris–HCl, pH 8.0) followed by treatment with 100 μg/ml lysozyme and sonication on ice. Expression of 1-cysPrx-P1 protein was verified by Simply Blue™ SafeStain (Invitrogen) staining and Western blot. The protein obtained from bacteria without IPTG induction was used as negative control. Human 1-cysPrx with a C-terminal His tag also was prepared as described above and used as a positive control [2]. Rat 1-cysPrx [15] without a His tag was expressed in *E. coli*.

2.9. Western blot

Mouse tissues were homogenized in lysis buffer containing added protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and 10 μg/ml leupeptin) followed by sonication on ice. Protein concentrations were estimated using the Bradford assay (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard. Protein samples were subjected to electrophoresis on 12% Novex Tris-Glycine Gels (Invitrogen) and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). For reduced gels, 2.8 mM β-mercaptoethanol was added to the samples. For blocking, membranes were incubated for 1 h or overnight at 4°C in blocking solution, 0.1% Tween 20–TBS buffer containing 5% non-fat dry milk. After washing, the membranes were probed with mouse anti-1-cysPrx monoclonal antibody, 1:2000, or rabbit anti-1-cysPrx polyclonal anti-peptide antibody, 1:3000, followed by horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia, Piscataway, NJ, USA), 1:3000. The monoclonal antibody was generated against recombinant human protein [15] while the polyclonal anti-peptide antibody was generated to a synthetic peptide (amino acids 196–211 of the human protein) [6]. The reaction was detected by chemiluminescence using ECL Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ, USA) and exposed to X-Omat AR-2 X-ray film (Eastman Kodak Company, Rochester, NY, USA).

3. Results

3.1. Viability of null mice

The mating of mice heterozygous for the 1-cysPrx null allele produced 17 wild type mice (+/+), 30 heterozygous mice (+/−) and 16 null mice (−/−), very close to the expected Mendelian ratio of 1:2:1, indicating that the null mice were viable. The null mice did not show any obvious physical differences from their +/+ and +/− littermates. The level of expression of the 1-cysPrx gene in lungs of heterozygous mice was around 50% of the level found in homozygous wild type mice as determined at the mRNA level by real-time PCR (Fig. 1D) and at the protein level by Western blot (Fig. 1E). Both mRNA and protein for 1-cysPrx were undetectable in the lungs of the knock-out mice (Fig. 1D,E). Null mice were mated and produced litters of null mice, indicating that both null males and null females were fertile.

3.2. Specificity of primers for 1-cysPrx and 1-cysPrx-P1

Specific primers were designed in order to distinguish the cDNA sequence of 1-cysPrx and 1-cysPrx-P1 by RT-PCR (Fig. 2A). There are three and five nucleotide differences in the forward and reverse primers, respectively. The 248 bp RT-PCR product for 1-cysPrx contains a *Taq*I enzyme site that, when cleaved, results in two bands in the agarose gel, 152 bp and 96 bp; there is no *Sma*I enzyme site. In contrast, the 248 bp RT-PCR product for 1-cysPrx-P1 contains a *Sma*I enzyme site that, when cleaved, results in two bands, 187 bp and 61 bp; there is no *Taq*I enzyme site (Fig. 2B). These differences facilitate the identification of the two RT-PCR products.

3.3. Expression of 1-cysPrx-P1 and 1-cysPrx in mouse tissues

Tissues obtained from male C57BL/6J mice were used to measure the expression of 1-cysPrx-P1 and 1-cysPrx by both real-time PCR and Western blot. The presence of 1-cysPrx mRNA was detected in all tissues examined including liver, kidney, heart, testis, brain, seminal gland, skeletal muscle, spleen, and small intestine with the highest expression level in lung (Fig. 3A). On the other hand, 1-cysPrx-P1 mRNA was expressed only in mouse testis (Fig. 3B) with an expression level that was ~25-fold higher than that for 1-cysPrx (Fig. 3C). Western blot results using the polyclonal anti-peptide antibody showed protein levels consistent with the results for 1-cysPrx mRNA expression (Fig. 3D).

3.4. Immunoreactivity of recombinant 1-cysPrx-P1

We utilized several antibodies to 1-cysPrx to confirm the pattern of expression in tissues. To determine immunoreactiv-

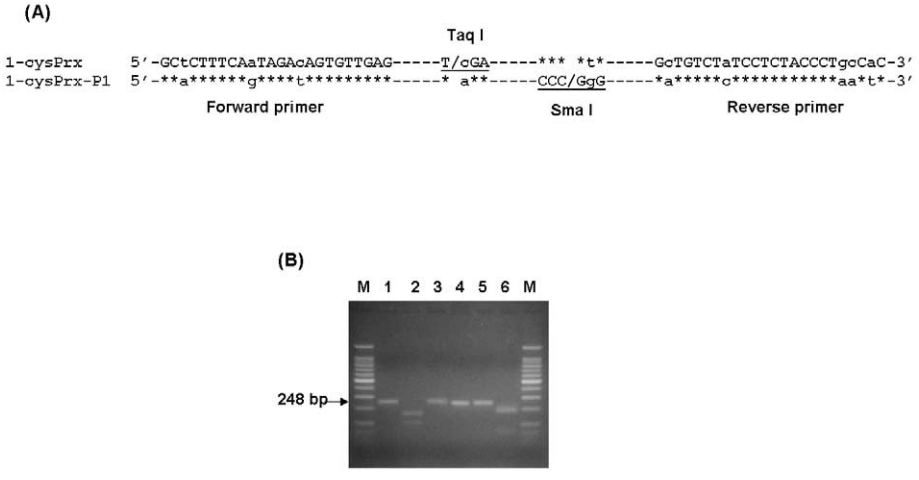

Fig. 2. Specificity of primers for 1-cysPrx and 1-cysPrx-P1. A: Comparison of primer sequences for 1-cysPrx and 1-cysPrx-P1 and restriction endonuclease sites. The nucleotides that are different in the two sequences are indicated by a lower case letter and nucleotides that are identical are indicated by an asterisk. The 1-cysPrx PCR product contains a *Taq*I enzyme site, but no *Sma*I enzyme site. The 1-cysPrx-P1 PCR product contains a *Sma*I enzyme site, but no *Taq*I enzyme site. B: Primer-specific PCR products as detected by RT-PCR followed by restriction endonuclease digestion. Lane M, 100 bp DNA ladder (Promega). Lanes 1–3, expression of 1-cysPrx in mouse lung; lanes 4–6, expression of 1-cysPrx-P1 in mouse testis; lanes 1 and 4, no enzyme digestion; lanes 2 and 5, *Taq*I digestion; lanes 3 and 6, *Sma*I digestion.

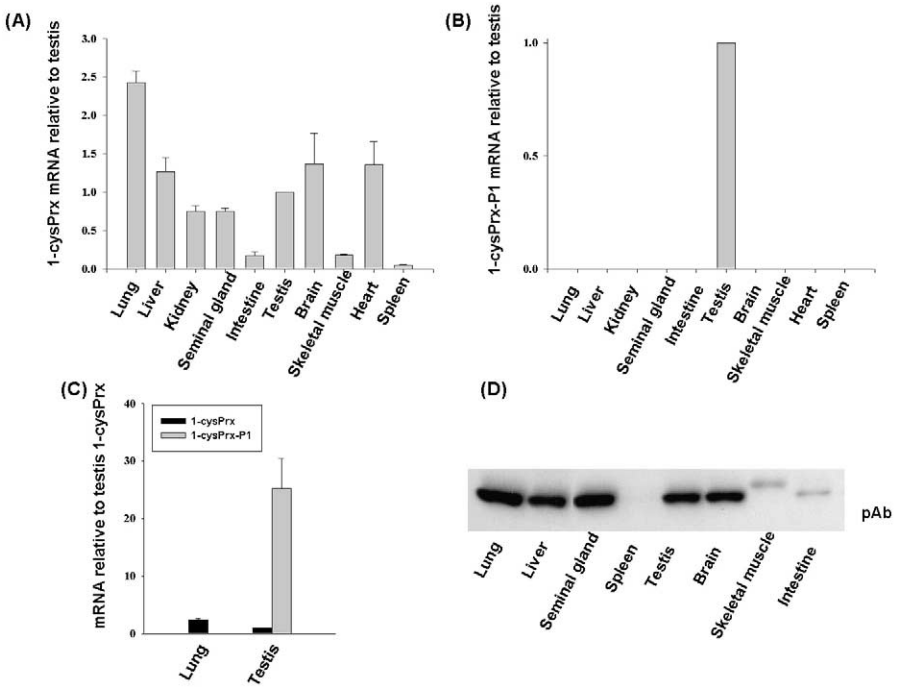

Fig. 3. Expression of 1-cysPrx and 1-cysPrx-P1 in mouse tissues. Expression of 1-cysPrx (A) and 1-cysPrx-P1 (B) was measured by real-time PCR as described in Section 2. C: Comparison of 1-cysPrx and 1-cysPrx-P1 mRNA levels in mouse lung and testis. In A–C results are mean ± S.E.M. for three mice and the expression level of 1-cysPrx (A,C) or 1-cysPrx-P1 (B) in testis was used as control (ratio = 1). D: Western blot of mouse tissues from a reduced gel (10 μg protein per lane) using the polyclonal anti-peptide antibody.

ity of the antibodies to 1-cysPrx-P1, this protein was synthesized using a bacterial expression vector. Protein synthesis required IPTG induction (Fig. 4A). The expressed protein reacted to the anti-1-cysPrx monoclonal antibody (Fig. 4B), but not to the anti-1-cysPrx polyclonal anti-peptide antibody (Fig. 4C). The lack of reactivity of the polyclonal anti-peptide antibody to 1-cysPrx-P1 is consistent with the difference in six out of the 16 amino acids compared to the human sequence which was the basis for the immunizing peptide. The wild type mouse protein had a difference of only two amino acids from the immunizing peptide which may account for its reactivity to the antibody. Thus, the immunoblots in Fig. 3D using polyclonal anti-peptide antibody reflect only 1-cysPrx while the monoclonal antibody is required to detect 1-cysPrx-P1 protein.

3.5. *Lack of 1-cysPrx-P1 protein expression in 1-cysPrx knock-out mouse*

Analysis of lungs and testis of 1-cysPrx knock-out mice was used to evaluate whether 1-cysPrx-P1 mRNA generated a protein in vivo. As expected, the knock-out animals showed no expression of 1-cysPrx mRNA that was detectable by RT-PCR (Fig. 5A) or protein that was detectable by Western blot using either the monoclonal or polyclonal anti-peptide antibody (Figs. 1E and 5C). On the other hand, the mRNA level of 1-cysPrx-P1 in the testis of 1-cysPrx knock-out mice was not significantly different ($P > 0.05$) than that for wild type mice (Fig. 5A,B). Despite the presence of large quantities of mRNA, protein expression was not detected in the testis of 1-cysPrx knock-out mouse (Fig. 5C). Neither mRNA nor protein for 1-cysPrx-P1 was detected in the lungs of 1-cysPrx knock-out mice.

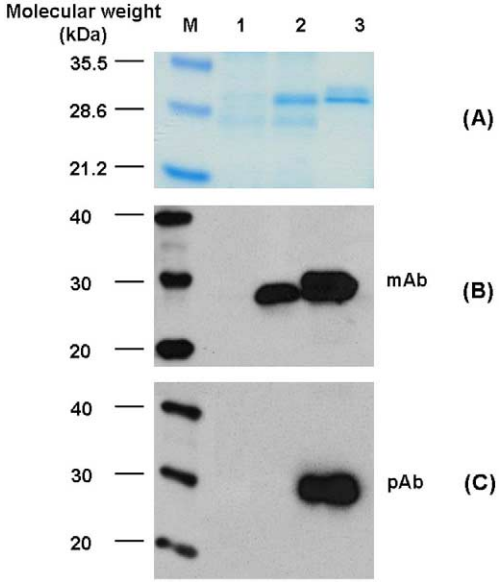

Fig. 4. Immunoreactivity of recombinant 1-cysPrx-P1 protein. For this analysis, 10 μg protein was loaded in each lane. A: Expressed 1-cysPrx-P1 recombinant protein stained by Simply Blue SafeStain (Invitrogen). B,C: Western blot with anti-1-cysPrx monoclonal antibody (B) and anti-1-cysPrx polyclonal anti-peptide antibody (C). Lane M, prestained sodium dodecyl sulfate–polyacrylamide gel electrophoresis standards (Bio-Rad) (A), and MagicMark Western standards (Invitrogen) (B,C); lane 1, uninduced protein in the absence of IPTG in the culture medium; lane 2, 1-cysPrx-P1 protein expression was induced by adding 1 mM IPTG to the culture medium; lane 3, bacterial expressed human 1-cysPrx with C-terminal His tag was used as a positive control.

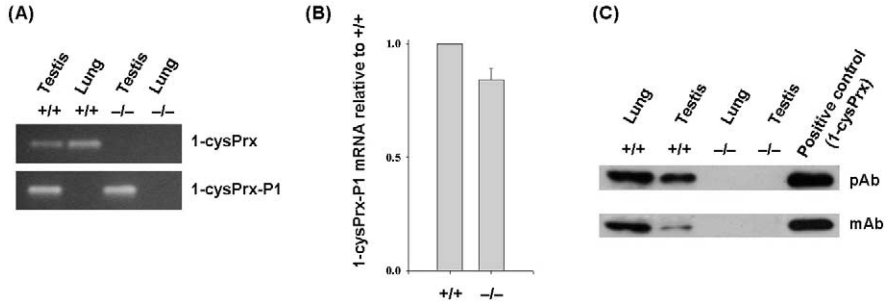

Fig. 5. Expression of 1-cysPrx and 1-cysPrx-P1 in wild type and 1-cysPrx knock-out mice. Expression of 1-cysPrx and 1-cysPrx-P1 was studied by RT-PCR (A), real-time PCR (B), and Western blot (C) in mouse lung and testis as described in Section 2. B: The expression of 1-cysPrx-P1 in homozygous wild type mice was used as the control (ratio = 1). C: The positive control is bacterially expressed rat 1-cysPrx protein. Each lane was loaded with 30 μg protein.

4. Discussion

Although peroxiredoxins are found in organisms from all kingdoms, individual proteins have unique properties and tissue and organelle distribution [16]. There are six mammalian isoforms; 1-cysPrx is the only member that contains one conserved cysteine residue while other members (Prx I–V) contain two conserved cysteine residues. 1-cysPrx in rat tissues is expressed at its highest mRNA and protein levels in the lung [15–17]. In the present study of mouse tissues, the highest level of 1-cysPrx expression also was found in the lung. The present results with real-time PCR are consistent with a previous analysis of mouse tissue by Northern blot [8]. However, a report using real-time PCR found that liver was the organ with the highest 1-cysPrx expression level [18]. This latter report used β-actin mRNA level to normalize the tissue results. Previous reports have indicated that the liver content of β-actin is significantly lower than many other tissues [19,20] and we have found by real-time PCR of mouse organs (data not shown) that the lung content of β-actin mRNA is approximately five-fold greater than liver content. Thus, normalization to β-actin mRNA does not reflect the true relationship between liver and lung with respect to the content of 1-cysPrx mRNA.

As expected, there is no mRNA or protein corresponding to 1-cysPrx in the null mice. In the present study, we investigated the possible expression of the very similar intronless 1-cysPrx-P1 gene and whether it could be induced by the absence of 1-cysPrx protein. Our findings indicate that the mRNA for this intronless gene in both wild type and knock-out animals is found only in testis. The findings in the wild type mouse are consistent with results of a previous study [21]. To study possible expression of the protein, we used a monoclonal antibody which reacts to expressed recombinant 1-cysPrx-P1 protein and evaluated the knock-out mouse where there is no background from the 1-cysPrx gene. Corresponding to the lack of mRNA, there was no expression of 1-cysPrx-P1 protein in the lung. Although the protein could have been degraded during preparation for Western blot, this is unlikely since protease inhibitors were used during tissue disruption and 1-cysPrx was intact. Even in testis where the level of 1-cysPrx-P1 mRNA is much higher than the level of the 1-cysPrx mRNA, we were unable to detect 1-cysPrx-P1 protein.

These results suggest that 1-cysPrx-P1 is an mRNA-expressed pseudogene that does not result in protein expression in vivo. Several other genes which express mRNA, but not protein, have been described and considered as pseudogenes including human L-threonine 3-dehydrogenase gene [22], mouse hnRNP A2 pseudogene [23], and mouse Makorin1-p1 [24]. The presence of messenger RNA for 1-cysPrx-P1 but the absence of protein suggests translational control of its expression. However, it is possible that 1-cysPrx-P1 protein is expressed in the testis but rapidly degraded so that its steady-state tissue content is below the level that can be detected by Western blot.

The highly conserved nature of 1-cysPrx and the finding that blocking its expression is lethal in at least one cell line [7] suggested that it might be indispensable for survival. The data indicate that, at least in mice, knocking out the 1-cysPrx gene does not grossly compromise development or fertility. However, the knock-out mice do show significantly increased sensitivity to the toxic effects of exposure to 100% O_2 (manuscript in preparation). While these studies were proceeding, another laboratory knocked out the 1-cysPrx gene using a different targeting strategy, obtaining similar results in terms of mouse viability. The latter mice showed increased sensitivity to the toxic effects of the herbicide paraquat [18]. Thus, mice with a knock-out of 1-cysPrx show a phenotype of increased sensitivity to oxidant stress, although they do not appear to show oxidative lung injury during their initial 3 months of life under unstressed conditions. This result is not unexpected since there is considerable redundancy of anti-oxidant enzymes and a similar lack of effect under unstressed conditions has been described for knock-out of cytosolic glutathione peroxidase [25,26] and CuZn superoxide dismutase, although the latter condition did result in altered fertility and fetal viability [27]. The present study shows that 1-cysPrx-P1 is not required for the compensatory process that protects mice from oxidant injury under unstressed conditions.

In summary, this study demonstrates in mice that: (1) 1-cysPrx is expressed in liver, kidney, heart, testis, brain, seminal gland, skeletal muscle, spleen, and small intestine, with the highest level of expression in lung; (2) inactivation of the 1-cysPrx gene is not lethal and does not affect fertility; (3) 1-cysPrx-P1 mRNA is expressed only in testis; and (4) 1-cysPrx-P1 protein is not detectable in the testis of 1-cysPrx knock-out mouse. These data indicate that 1-cysPrx-P1 is an mRNA-expressed pseudogene.

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