Mutation of Serine 32 to Threonine in Peroxiredoxin 6 Preserves Its Structure and Enzymatic Function but Abolishes Its Trafficking to Lamellar Bodies*

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Peroxiredoxin 6 (Prdx6), a bifunctional protein with phospholipase A₂ (aiPLA₂) and GSH peroxidase activities, protects lungs from oxidative stress and participates in lung surfactant phospholipid turnover. Prdx6 has been localized to both cytosol and lamellar bodies (LB) in lung epithelium, and its organellar targeting sequence has been identified. We propose that Prdx6 LB targeting facilitates its role in the metabolism of lung surfactant phosphatidylcholine (PC). Ser-32 has been identified as the active site in Prdx6 for aiPLA₂ activity, and this activity was abolished by the mutation of serine 32 to alanine (S32A). However, aiPLA₂ activity was unaffected by mutation of serine 32 in Prdx6 to threonine (S32T). Prdx6 protein expression and aiPLA₂ activity were normal in the whole lung of a “knock-in” mouse model carrying an S32T mutation in the Prdx6 gene but were absent from isolated LB. Analyses by proximity ligation assay in lung sections demonstrated the inability of S32T Prdx6 to bind to the chaperone protein, 14-3-3e, that is required for LB targeting. The content of total phospholipid, PC, and disaturated PC in lung tissue homogenate, bronchoalveolar lavage fluid, and lung LB was increased significantly in Prdx6-S32T mutant lungs, whereas degradation of internalized [3H]dipalmi-
sotropic, where it serves as a vital cellular antioxidant (18). We have shown recently that both the GSH peroxidase and PLA₂ activities of the GSH peroxidase activity is able to reduce phospholipid hydroperoxides, and Prdx6 is the only mammalian peroxiredoxin known with the ability to both hydrolyze and reduce phospholipids (9–11). The protein is widely expressed in tissues with especially high levels in the lung (9). Within the lung, Prdx6 is expressed at relatively high levels within alveolar type II epithelial (ATII) cells where it has been localized to both cytosol and acidic compartments (LB and lysosomes) (12, 13). Prdx6 in LB plays a key role in the degradation and resynthesis of surfactant DPPC (14, 15). Our previous studies showed that overexpression of Prdx6 led to increased metabolism of DPPC in lungs and ATII cells (16), whereas Prdx6 null mice exhibited DPPC accumulation in lung tissue and LBs (17); these results are compatible with an important role for aiPLA₂ activity in lung phospholipid turnover. However, the greatest fraction of Prdx6 is not in LB but is cytosolic, where it serves as a vital cellular antioxidant (18). We have shown recently that both the GSH peroxidase and PLA₂ activities of Prdx6 play important roles in the repair of peroxidized lung cell membranes (19).

Lung surfactant is a phospholipid-protein complex that is secreted by lung epithelium and is essential to maintain alveolar stability for normal lung function. The phospholipids of lung surfactant are synthesized by alveolar type II (ATII) cells and stored in lamellar bodies (LB) for secretion into the alveolar space. LB are characterized by lysosome-related organelles (LRO) that are known to participate in biosynthetic pathways as illustrated by the synthesis of melanin by melanosomes, another LRO (1, 2). LB maintain an acidic pH and contain at least some of the enzymes that are required for degradation as well as synthesis of phospholipids (3–5). Changes in surfactant phospholipid composition have been linked to alterations in the biophysical properties of surfactant and may contribute to the development of lung disease (4–8).

Peroxiredoxin 6 (Prdx6) is a bifunctional enzyme with lysosome-type Ca²⁺-independent phospholipase A₂ (aiPLA₂) and GSH peroxidase activities; the GSH peroxidase activity is able to reduce phospholipid hydroperoxides, and Prdx6 is the only mammalian peroxiredoxin known with the ability to both hydrolyze and reduce phospholipids (9–11). The protein is widely expressed in tissues with especially high levels in the lung (9). Within the lung, Prdx6 is expressed at relatively high levels within alveolar type II epithelial (ATII) cells where it has been localized to both cytosol and acidic compartments (LB and lysosomes) (12, 13). Prdx6 in LB plays a key role in the degradation and resynthesis of surfactant DPPC (14, 15). Our previous studies showed that overexpression of Prdx6 led to increased metabolism of DPPC in lungs and ATII cells (16), whereas Prdx6 null mice exhibited DPPC accumulation in lung tissue and LBs (17); these results are compatible with an important role for aiPLA₂ activity in lung phospholipid turnover. However, the greatest fraction of Prdx6 is not in LB but is cytosolic, where it serves as a vital cellular antioxidant (18). We have shown recently that both the GSH peroxidase and PLA₂ activities of Prdx6 play important roles in the repair of peroxidized lung cell membranes (19).

Using a protein truncation approach, we have previously identified the Prdx6 organellar targeting motif as a sequence comprising amino acids 31–40 (DSWGLFLSP40) that is located in the N-terminal region of the protein (13). The serine at position 32 (Ser-32) of Prdx6 is essential for its targeting to lamellar bodies and lysosomes. Thus, mutation of Ser-32 to alanine abolished Prdx6 organellar localization in A549 and

DPPC, dipalmitoylphosphatidylcholine; DSPC, disaturated phosphatidylcholine; LB, lamellar body; LRO, lysosome-related organelles; lyso-PC, lysophosphatidylcholine; MPMVEC, mouse pulmonary microvascular endothelial cells; PC, phosphatidylcholine; PLPCOOH, 1-palmityl, 2-linoleoyl, sn-3-phosphocholine hydroperoxide; EGFP, enhanced GFP.
ML12 cells, models of ATII cells derived from human and mouse, respectively (13). Ser-32 also is required for binding of Prdx6 to its lipid substrates and constitutes an important component of the catalytic triad (His-26–Ser-32–Asp-140) that is required for the PLA2 activity of the protein; thus, the S32A mutant does not exhibit PLA2 activity or reduction of phospholipid hydroperoxides (20). Prdx6 organellar localization does not depend on binding of the protein to phospholipids (13) but does depend on binding to a chaperone molecule, 14-3-3 (21).

In this study, a unique “knock-in” mouse model carrying serine 32 to threonine mutation in the Prdx6 gene was used as a model of targeted depletion of PLA2 in LB. Our study shows that threonine can substitute for serine for the enzymatic activities of the protein but not for Prdx6 targeting to LB. Evaluation of surfactant phospholipid metabolism in lungs from Prdx6-S32T knock-in mice confirms an important role for lamellar body Prdx6 in the degradation and remodeling of lung surfactant phosphatidylcholine.

**Experimental Procedures**

**Recombinant Proteins**—The cloning of human codon-optimized Prdx6 cDNA and its insertion into pJexpress414 vector, its expression in *Escherichia coli*, and its purification by ion-exchange chromatography have been described previously (10, 23). For generation of recombinant mutant proteins, complementary mutagenic oligonucleotide pairs were purchased from Eurofins MWG Operon (Huntsville, AL). To generate the Prdx6-S32T mutant construct, the oligonucleotide primers were as follows: 5’-CTTCTAGACCTTCCTGGGCATTACC-GAGGAGCATCCT-3′ (forward) and 5’-AGGATCCCAGGT-TATCGCGCAAGAATCTCAGAAG-3′ (reverse). Underlined bases represent the mutated codon. Cells were expressed in the BL21-DE3 host strain of *E. coli* (Novagen, Madison, WI), and recombinant proteins were purified using ion-exchange chromatography (20, 24, 25).

**Cell Lines**—The human lung carcinoma A549 and human renal embryocarcinoma 293T cell lines were obtained from ATCC. A549 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and antibiotics. These cells are used as a model for ATII cells as their lysosomes exhibit some of the characteristics of LB (26). 293T cells were propagated in DMEM supplemented with 10% FBS (Sigma), 2 mm l-glutamine, and 1 mm sodium pyruvate (Invitrogen) and were used for lentivirus production. For transient expression of GFP-tagged constructs, A549 cell layers at 95% confluence in 6-well plates were transfected with 3 μg of each expression plasmid in 10 μl of Lipofectamine 2000 reagent (Invitrogen) per well according to the manufacturer’s protocol. The mammalian expression plasmids encoding full-length Prdx6 with an N-terminal green fluorescent protein (GFP) tag and the GFP-Prdx6 (S32A) mutant have been described previously (13). Generation of the GFP-Prdx6 (S32T) mutation was performed similarly using the QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA), according to the manufacturer’s instructions. Generation of the GFP-Prdx6 mammalian expression plasmids used the following HPLC-purified primers: 5’-CAGGATTTCTAGAGATACATGGGGCAT-ATTCTTTTTC-3′ (forward) and 5’-GAAAAGAATGCCCAGTATCTCTAGAATCGTG-3′ (reverse). The underlined bases represent the mutated codon. Cells were subjected to experimental treatments at 48 h after transfection.

Mouse pulmonary microvascular endothelial cells (MPMVEC) were isolated from mouse lungs using enzymatic digestion and differential adherence as described previously (27–29). MPMVEC were propagated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), non-essential amino acids, and antibiotics. Cells were maintained under static culture conditions in 5% CO2 at 37 °C.

**Lentivirus Vector Construction and Transfection**—A third-generation lentivirus vector was used to construct particles for delivery of wild type (WT) or mutant Prdx6 to MPMVEC. HMD control lentivirus transfer plasmid, pCMV-dR8.2 packaging plasmid, and pCMV-VSVG envelope plasmid were the generous gifts from Dr. Mitchell Weiss (The Children’s Hospital of Philadelphia, PA). The human WT Prdx6 coding region was subcloned into HMD lentivirus transfer vector as an XhoI-EcoRI fragment. Serine 32 mutations in Mice-HMD-Prdx6 were generated by overlapping PCR using a pair of forward and reverse primers. The HMD-Prdx6/S32T lentivirus transfer plasmids were generated by the following primers: 5’-CAGGACTTTCTGGGAGCATGCCGATGCTCTCTTC-3′ (forward) and 5’-GAGGAAGAGATGCCCATGCTCTCCCAAGAAGTCTG-3′ (reverse). For the HMD-Prdx6/S32A mutant transfer plasmid, the primers were 5’-CAGGACTTTCTGGGAGAAGCAGTGCTCTTC-3′ (forward) and 5’-GAGGAAGAGATGCCCATGCTCTCCCAAGAAGTCTG-3′ (reverse). The underlined bases represent the mutated codon.

Lentivirus particles were produced by co-transfection of one of three transfer vector plasmids (HMD, HMD-Prdx6/S32T, or HMD-Prdx6/S32A transfer vector plasmid) with the packaging plasmid (pCMV-dR8.2) providing the enhanced green fluorescent protein (EGFP) viral gene, and the envelope plasmid (pCMV-VSVG) encoding the vesicular stomatitis virus glycoprotein. The plasmids were transfected into HEK 293T cells using Lipofectamine 2000 transfection reagent (Invitrogen) according to the recommended protocol of the manufacturer. Conditioned medium was collected 48 and 72 h after transfection and stored at −80 °C. The virus titer was determined by infecting Prdx6 null MPMVEC (5 × 105) with serially diluted virus stocks and quantifying the numbers of EGFP-positive cells.

To study the effects of S32A and S32T mutations in cells, Prdx6 null MPMVEC were incubated for 72 or 96 h with wild type or Ser-32 mutant Prdx6 virus-containing supernatants plus added Polystyrene (8 μg/ml). Empty HMD lentiviral transfer plasmid and HMD lentiviral transfer plasmid containing WT Prdx6 were used as negative and positive controls, respectively. Infection efficiency was determined by the percent of EGFP-positive cells. At the end of the incubation, cells were lysed in...
1× cell lysis buffer (Cell Signaling Technology, Danvers, MA), and the lysate was used for enzymatic assays.

**Animals**—Constructs for generation of Prdx6 S32T knock-in mice were developed, and mice were generated by the Gene Targeting Core and Laboratory and the Transgenic and Chimeric Mouse Facility of the University of Pennsylvania (Fig. 1A). To retrieve the part of the Prdx6 gene to be mutated, short homologous arms for the pL253 retrieval vector (National Institutes of Health, NCI-Frederick, recombineering website) were amplified from a Prdx6 gene-containing BAC clone from a C57Bl/6J genomic library; this clone was used as all of our previous mouse studies have used the C57Bl/6J strain. Sequences of 300 bp were amplified by PCR to generate linear fragments flanked by either NotI/HindIII or HindIII/SpeI, respectively, and then co-ligated into the pL253 MCS NotI and SpeI sites. The resulting construct was linearized using HindIII and transfected into E. coli SW102 containing heat shock-inducible Red recombination proteins and the Prdx6 BAC clone. Following treatment at 42 °C for 15 min, ampicillin-resistant colonies were screened for homologous recombination at the 300-bp flanking regions. pL253 derivatives with an 12-kb fragment containing exons 1–4 were selected.

To generate the S32T mutant allele, first a mini-vector was constructed. An 300-bp-long genomic Prdx6 fragment located ~150 nucleotides upstream of exon 1 was generated by PCR, together with an ~700-bp Prdx6 PCR fragment containing exon 1 located just downstream of the ~300-bp fragment. The genomic PCR primers were designed to introduce the appropriate restriction enzyme sites at the ends of the fragments, for cloning into PL451 (National Institutes of Health, NCI-Frederick, recombineering website). The two PCR fragments were ligated into PL451 by two subsequent ligation and cloning cycles. This pl451 derivative was mutated by site-directed mutagenesis at codon Ser-32 (TCG to ACG) in exon 1 to

FIGURE 1. Generation of Prdx6 S32T mutant mice. A, targeting vector was constructed with a flippase recombinant target-flanked neo-cassette 290 bp downstream of exon 1 and electroporated into C57B16 ES cells. Neo-positive ES clones were screened by Southern blotting using a probe located just upstream of the 5’ end of the vector. B, probe was used for Southern analysis of ApaI cut genomic DNA, and positive ES clones yielded the expected 14.9-kb WT band and 16.7-kb mutant band, due to insertion of the neo-cassette. C, schematic of targeted mutation of endogenous Prdx6. Serine to threonine mutation creates a new restriction site for the SnaB1 enzyme. Red represents the Ser to Thr mutation, and purple indicates the DNA coding for the untranslated regions of Prdx6 mRNA. Tg, transgenic. D, genotyping by PCR + SnaB1 digestion (WT = 341 bp only; mutation = 270 + 71 bp) showing wild type (WT), homozygous (KI), and heterozygous (HET) mice.
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derive the final mutagenic mini-targeting vector. This mini-vector, which also contained a pgk EM7 neo poly(A) cassette flanked by FLP recombinase target sites, was linearized, transformed, and recombined into the above ~12-kb Prdx6 vector backbone by heat induction of the λRed recombination enzymes, as above.

Generation of the final targeting vector with the S32T mutation was by neomycin/kanamycin resistance using 50 µg/ml kanamycin. This final targeting construct was linearized, sequence-verified, and electroporated into C57BL/6J ES cells (EAP6 ES cells) for insertion of the mutant sequences into the mouse genome by homologous recombination. ES clones showing homologous recombination were identified by Southern blotting (Fig. 1B). These clones were karyotyped; the Ser-32 mutation was verified by genomic sequencing and used for blastocyst injection into CD-1/BALB/c mice. The serine to threonine mutation creates a convenient restriction endonuclease site for the SnaBI enzyme that is otherwise absent from the WT sequence (Fig. 1C). Successful transmission of the mutation was detected by PCR amplification of the region around the mutation followed by digestion of the PCR product with the SnaBI restriction enzyme. The wild type allele displays one large band, and the mutant allele displays two smaller bands (Fig. 1D).

Mice were bred and maintained in the animal care facilities of the University of Pennsylvania (Philadelphia). S32T-Prdx6 knock-in mice developed normally and showed no obvious physical differences from their littermates. Wild type C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Both male and female 8–10-week-old mice were used for experiments. All animal protocols were reviewed and approved by the University of Pennsylvania Animal Care and Use Committee. Animals were housed under the National Institutes of Health and United States Drug Administration guidelines for the care and use of animals in research.

Lung Morphology—Lungs were excised from wild type (WT) or S32T mutant mice, cleared of blood, and fixed by perfusion through the pulmonary artery with 4% paraformaldehyde (30, 31). Tissue samples for routine morphology were processed by the Pathology Core of The Children’s Hospital of Philadelphia (Abramson Research Center, Philadelphia) and stained with hematoxylin and eosin (H&E). Lung sections were examined by three independent observers, and randomly selected fields were chosen for comparison.

Isolation of Lamellar Bodies—Mouse lungs that had been cleared of blood and subjected to bronchoalveolar lavage were homogenized, and lamellar bodies were isolated by upward flotation in a sucrose density gradient (17, 32). This method produces a relatively pure population of largely intact lamellar bodies with a phospholipid to protein ratio of ~10.

Circular Dichroism Measurement—Circular dichroism (CD) measurements of wild type and mutant proteins (2.5 µM in 40 mM potassium phosphate buffer, pH 7.4) were carried out by the Protein and Proteomic Core Facility of The Children’s Hospital of Philadelphia. Spectra were recorded with a Jasco J810 circular dichroism spectropolarimeter (Jasco Analytical Instruments, Easton, MD). The output of the CD spectrometer was analyzed and recalculated according to the protein concentration, amino acid content, and cuvette thickness into molecular ellipticity units (degrees/cm²/dmol) using Jasco’s SpectroManager software (version 2.8.1.1).

Enzymatic Activity Assays—Enzymatic activities were measured in recombinant proteins, lung homogenates, LB, or lysed MPMVEC. PLₐ activity was measured at pH 4 by radiochemical detection of liberated fatty acid as described previously (15, 33). The substrate was unilamellar liposomes consisting of DPPC, egg PC, cholesterol, and phosphatidylglycerol in the molar ratio of 50:25:15:10 that were prepared by extrusion through a membrane under pressure (15). This lipid mixture was chosen to reflect the lipid composition of lung surfactant (34). Liposomes were labeled with 1-palmitoyl, 2-[9,10-³H]palmitoyl, sn-glycerophosphorylcholine ([³H]DPPC) at a specific activity of 2 mCi/µmol DPPC. Analysis by dynamic light scattering (DLS 90 Plus Particle Size Analyzer, Brookhaven Instruments, Holtsville, NY) showed a homogeneous population of liposomes that was 100–120 nm in diameter and represented >95% of total vesicles. Authentic lipids were purchased from Avanti Polar Lipids (Alabaster, AL).

Radiochemicals were purchased from PerkinElmer Life Sciences. Peroxidase activity was determined by measuring the initial slope of the decrease in NADPH fluorescence with time in the presence of glutathione (GSH) and GSH reductase. The substrate was either H₂O₂ or 1-palmitoyl,2-linoleoyl, sn-phosphatidylcholine hydroperoxide (PLPCOOH) in 40 mM PBS, pH 7.4, with 5 mM EDTA and 1 mM NaNO₂. 0.1% Triton X-100 was added for assay with PLPCOOH as substrate (35). Assay for the recombinant protein was performed in the presence of πGST equimolar to Prdx6. πGST was generated using a plasmid supplied by Dr. Roberta Colman (University of Delaware) and purified as described previously (36). PLPCOOH was prepared by enzymatic oxidation of PLPC as described previously (35). Protein concentration was measured using the Bradford protein assay with bovine γ-globulin as standard (Bio-Rad).

Lung Perfusion—Perfusion of isolated lungs was carried out as described previously (17, 37). Mice were anesthetized with intraperitoneal ketamine/xylazine/acepromazine (10:15:2 mg/kg body weight). The abdomen and chest of the anesthetized and continuously ventilated mouse (60 cycles/min and 0.3–ml tidal volume with 5% CO₂ in air) were incised, and the lungs were cleared of blood by perfusion with Krebs-Ringer bicarbonate solution supplemented with 3% fatty acid-free BSA and 10 mM glucose (supplemented KRB). Lungs were placed in the lung perfusion chamber, continuously ventilated as above, and perfused at 2 ml/min.

Lung Lipid Content and Uptake and Degradation of DPPC—The bronchoalveolar lavage fluid (BALF), post-lavage lung homogenate, and isolated LB were analyzed for total phospholipid and PC and disaturated PC (but consisting primarily of DPPC) fractions as described previously (14, 15, 16, 17). Total phospholipids were extracted by the Bligh and Dyer procedure (38), and PC was isolated by thin layer chromatography (TLC). DSPC was separated from total phosphatidylcholine (PC) by treatment with OsO₄ followed by separation on a neutral alumina column. Fractions were quantitated by measurement of lipid phosphorus. The content of phospholipid fractions in
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BALF, lung homogenate, and LB was expressed relative to body weight, lung weight, and LB protein, respectively.

Uptake and degradation of DPPC in isolated mouse lungs during a 2-h perfusion was measured as described previously (14, 15, 33). Briefly, unilamellar “surfactant-like” liposomes labeled with [choline-methyl-3H]DPPC were instilled endotra- cheally in the anesthetized mouse, and lungs were isolated for recirculating perfusion. At the end of perfusion, lungs were lavaged to remove the remaining liposomes from the alveolar space and homogenized. Lungs treated similarly but without perfusion were used as the zero time value. Radioactivity was measured in the BALF, lung perfusate, and lung homogenate. For products of metabolism, total phospholipids and the aque- ous fraction were obtained from the Bligh and Dyer separation; total PC and lyso-PC were obtained by TLC separation, and DSPC was determined by separation from total PC as above; unsaturated PC was calculated as total PC minus DSPC.

DPPC uptake was calculated from the initial alveolar disintegrations/min (dpm) minus dpm recovered in lung lavage (plus perfusate) and expressed as a percent of the instilled dpm. The initial alveolar dpm was estimated from the instilled dpm and the alveolar DSPC pool size. Disintegrations/min was meas- ured in the unsaturated PC, lyso-PC, and aqueous fractions and expressed as percent of internalized DPPC (uptake). Uptake as calculated from the reduction of dpm in the alveolar space versus the sum of dpm recovered in total PC plus meta- bolic products (data not shown) were approximately equal indi- cating reliability of the methodology, as described previously (17).

Immunoﬂuorescence—To evaluate Prdx6 localization in A549 cells that had been transfectected to express GFP-Prdx6, cells were cultured on glass coverslips, rinsed with PBS, and ﬁxed with cold ethanol/acetone (1:1 by volume) for 5 min on ice. For Prdx6 localization in lung tissue, lungs that were cleared of blood and perfused with ﬁxative as described above were inﬁltered with 2% low-melting temperature agarose (Sigma), and the gel was allowed to solidify on ice for 1 h. Lungs were dis- sectected, and the same lobe from WT and mutant lungs was sec- tioned with an oscillating tissue slicer (MRC 5000, Electron Microscopy Sciences, Hatﬁeld, PA). Cells or tissue sections were then incubated for 10 min (cells) or 30 min (tissue) with 1% Triton X-100 solution in PBS to maximally deplete Prdx6 from the cytosol followed by 1 h of blocking in 3% bovine serum albumin in PBS containing 0.2% Triton X-100.

Cells on coverslips were immunolabeled with a polyclonal (rabbit) antibody to lysosome-associated membrane protein 1 (LAMP-1) (Cell Signaling Technology, Danvers, MA) that was used as a marker for lysosome-like organelles. The primary antibodies used for subcellular localization in lung sections were the LAMP-1 antibody and a monoclonal antibody to Prdx6 (Chemicon EMD Millipore, Billerica, MA). Cells were incubated with primary antibody (1:200 dilution) in 0.2% Triton X-100 solution in PBS (T-PBS) for 1 h at room temperature; tissue sections were incubated with primary antibodies overnight at 4°C. After extensive washing with T-PBS, preparations were incubated for 1 h at room temperature with secondary Alexa Fluor-594-conjugated (red) goat anti-mouse IgG antibody (cells and tissue sections) and with Alexa Fluor-488-con-jugated (green) goat anti-rabbit IgG antibody (tissue sections only) (Molecular Probes, Eugene, OR) at 1:1,000 (cells) or 1:500 (tissue) dilution in T-PBS. After a ﬁnal extensive washing with T-PBS followed by PBS, the cells on coverslips or the tissue sections were mounted on slides with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Subcellular distribution of Prdx6 was observed by confocal microscopy (Radiance 2000; Bio-Rad) at ×600 magnification.

Co-localization of the LB and Prdx6 signals was quantitated by ImageJ software using the co-localization indices plug-in filter. The analysis selected the entire cell as the ﬁeld of interest and strictly followed the protocol provided by the plug-in filter. We calculated Manders’ co-localization coefﬁcient (39) that estimates co-localization of ﬂuorescence in the red and green channels; values can range from zero (no co-localization) to one (perfect co-localization). We also determined Pearson’s corre- lation coefﬁcient (39) that measures intensities of each channel for each pixel; values for co-localization range from −1 (perfect non-co-localization) to +1 (perfect co-localization). Values for these two indices were calculated for the total number of pixels in each image.

Duolink in Situ Proximity Ligation Assay—To detect the proximity between Prdx6 and the 14-3-3e chaperone molecule, we utilized the Duolink in situ proximity ligation assay (Olink Bioscience, Uppsala, Sweden) according to the manufacturer’s protocol. Lung tissue sections from WT or S32T mutant mice were processed as described above. Mouse monoclonal anti- body to Prdx6 (EMD Millipore) and rabbit polyclonal antibody to 14-3-3e (T-16, Santa Cruz Biotechnology, Santa Cruz, CA) were used to detect the proximity required for protein-protein interaction. Sections were immunolabeled overnight with pri- mary antibodies (1:100 dilutions in T-PBS) at 4°C. The pres- ence of the Duolink ﬂuorescence signal, which indicates that two proteins within the cell are separated by <40 nm, was observed by confocal microscopy at ×600 magnification.

Western Blot Analysis—Western blot analysis was performed using the two-color Odyssey LI-COR (Lincoln, NE) technique as described previously (27). A polyclonal antibody to Prdx6 was used (22) at a dilution of 1:1,500 in blocking buffer. A poly- clonal antibody made in rabbits was used to detect surfactant protein-A (40), and a mouse monoclonal antibody was used to detect GAPDH (EMD Millipore). Secondary antibodies were IrDye 800 goat anti-rabbit and IrDye 700 goat anti-mouse (Rockland, Gilbertsville, PA) for imaging on the green 800 nm and red 700 nm channels, respectively.

Statistical Analysis—Values are presented as means ± S.E. Statistical signiﬁcance was assessed with SigmaStat software (Jandel Scientiﬁc, San Jose, CA). Group differences were evalu- ated by one-way analysis of variance or by Student’s t test as appropriate. Differences between mean values were considered statistically signiﬁcant at p < 0.05.

Results

Effect of Serine 32 Site-specific Mutation on Secondary Structure and Enzymatic Activities of the Recombinant Prdx6 Protein—Our earlier study of the recombinant rat Prdx6 protein using circular dichroism (CD) spectral analysis indicated that serine 32 to alanine (S32A) mutation alters the secondary
structure of the protein with a markedly increased content of \( \alpha \)-helices and a decreased content of \( \beta \)-sheets (20). This earlier study also found that the S32A mutation led to the loss of aiPLA\(_2\) activity of the protein (20). The CD spectrum of recombinant human Prdx6 protein in the present study indicates a negligible effect of S32A mutation on protein secondary structure (Fig. 2). We interpret the different results between the previous and present studies as an indication of structural instability of the mutant protein. Nevertheless, consistent with our previous findings for the rat protein, serine 32 to alanine substitution in human Prdx6 resulted in the loss of aiPLA\(_2\) activity (Table 1). Peroxidase activity of the S32A mutated Prdx6 was not different from WT with \( \text{H}_2\text{O}_2 \) as substrate, but it was absent with PLPCOOH as substrate (Table 2). The loss of aiPLA\(_2\) and PLPCOOH peroxidase activities reflects the centrality of Ser-32 in the catalytic triad for aiPLA\(_2\) activity as well as the presence of Ser-32 in the putative phospholipid-binding site of the Prdx6 protein (13, 20).

Because of potential instability of Prdx6 associated with mutation of the Ser-32 to alanine, we evaluated a protein in which Ser-32 in human Prdx6 was mutated to threonine. The secondary structure of recombinant S32T Prdx6 as determined by CD spectral analysis indicated negligible difference from WT Prdx6 (Fig. 2). There also was no effect of this mutation on Prdx6 aiPLA\(_2\) activity (Table 1). Kinetic parameters for the PLA\(_2\) activity of WT and S32T-Prdx6 were investigated by varying DPPC concentrations. Double-reciprocal plots (Fig. 3) and the calculated Michaelis-Menten constant (\( K_m \)) and maximal velocity (\( V_{\text{max}} \)) for aiPLA\(_2\) activity (Table 1) were similar for the WT and threonine mutant proteins. Thus, S32T-Prdx6 recombinant protein fully retained its PLA\(_2\) enzymatic activity, in contrast to the S32A mutation. The S32T mutation also did not have any effect on the peroxidase activity of the recombinant Prdx6 protein with either \( \text{H}_2\text{O}_2 \) or PLPCOOH as substrate (Table 2).

Effect of Ser-32 Site-specific Mutations on Prdx6 Enzymatic Activities in Cells—To confirm our \textit{in vitro} findings and to study the effect of both Prdx6 S32A and S32T mutations in cells, we expressed human wild type or mutant protein in Prdx6 null MPMVEC using HMD lentivirus transfer vector. As detected by green fluorescent protein (GFP) expression, the efficiency for infection of Prdx6 null MPMVEC with human WT, S32A, and S32T mutant Prdx6 viruses was over 90% and equivalent for all constructs (Fig. 4A).

aiPLA\(_2\) and peroxidase activities in cell lysates were evaluated to determine the effect of \textit{Prdx6}-S32A and S32T mutations.
on enzymatic function of the endogenously synthesized protein. Similarly to the results with the recombinant protein, aiPLA₂ activity in lysates of MPMVEC cells transfected with WT or S32T Prdx6-lentiviruses was comparable, whereas the S32A mutation abolished the aiPLA₂ activity (Fig. 4B). Assay of peroxidase activity in the same MPMVEC cell lysates confirmed that the S32T mutation has no effect on Prdx6 enzymatic function with comparable levels of peroxidase activity using either H₂O₂ or PLPCOOH as substrate (Table 2). The significantly greater activity with H₂O₂ as substrate can reflect the presence of catalase and other H₂O₂ peroxidases besides Prdx6. However, “knock-out” of Prdx6 has shown that there are essentially no other phospholipid hydroperoxidases in lung cells so that reduction of PLPCOOH as determined by the peroxidase assay reflects cellular Prdx6 activity (41, 42). MPMVEC expressing S32A mutant protein showed a significant decrease of peroxidase activity with PLPCOOH as substrate, but there was no effect on activity with H₂O₂ substrate (Table 2). As described above, we interpret this substrate-related difference on the peroxidase activity of S32A-Prdx6 as reflecting an effect of the mutation on phospholipid binding.

Mutation of Serine 32 Abolishes Prdx6 Targeting to Lysosome-related Organelles in A549 Cells—Our previous studies have demonstrated Prdx6 expression in lysosomes, LB, and cytosol of lung epithelial cells (12). Within LB, Prdx6 was predominantly localized to the vesicular lumen (21). A 10-amino acid N-terminal sequence (31DSWGILFSHP40) was shown to determine Prdx6 organellar targeting (13). The serine at position 32 (Ser-32) is necessary for this subcellular distribution because the lamellar body localization of Prdx6 was abolished by mutation of its serine 32 to alanine (S32A) (13).

In this study, we evaluated protein targeting to lysosome-like organelles by generating GFP-tagged Prdx6 mutants with Ser-32 to alanine (S32A) or Ser-32 to threonine (S32T) mutation. The wild type A549 cells show small vesicular structures that stain positively for LAMP-1 and also for GFP-Prdx6 as indicated by the arrows in Fig. 5. As expected based on our previous results (13), the S32A-Prdx6 mutant also shows vesicular staining with LAMP-1, but there is relatively little corresponding GFP-Prdx6 staining (Fig. 5). Unexpectedly, because enzymatic activities were unaffected, the S32T-Prdx6 mutant also showed minimal GFP-Prdx6 vesicular staining. The decreased co-localization of GFP-Prdx6 and LAMP1 with both the S32A- and S32T-Prdx6 mutants is supported by the decreases in Manders’ overlap and Pearson’s correlation coefficients calculated from total pixels in Fig. 5 (Table 3).

Thus, both the S32A and S32T mutations result in a similar loss of Prdx6 organellar targeting in A549 cells (Fig. 5) despite their opposite results with respect to enzymatic activities (Tables 1 and 2). However, caveats associated with the co-localization procedure, namely the small size of the organelles and uncertainty regarding identity of the vesicles in A549 cells, the necessity to rely on transient transfection with subsequent variable levels of Prdx6 expression, and the requirement to deplete cytosolic Prdx6 prior to immunostaining, prevent a definitive conclusion regarding targeting of the protein. This led us to consider other approaches to evaluate the effect of S32T mutation on targeting of Prdx6 to LRO. To accomplish this, we produced and studied a S32T-Prdx6 knock-in mouse.

Characterization of Prdx6-S32T Mouse—Histological evaluation using H&E-stained sections indicated that the mutation of Ser-32 in Prdx6 to threonine had no observable effect on mouse lung morphology as compared with WT (Fig. 6). Evaluation of lung tissue sections from WT mice by immunofluorescence demonstrated the presence of Prdx6 in LB by its co-localization with LAMP-1 that was used as a marker of lysosome-related compartments (Fig. 7A). However, although lungs from S32T-Prdx6 mutant mice demonstrated Prdx6 fluo-
presence in epithelial cells, the fluorescence did not appear to be present in LB, i.e. organelles stained with LAMP-1 (Fig. 7B). The Manders’ overlap and Pearson’s correlation coefficients, calculated from total pixels in Fig. 7, A and B, indicate a marked decrease in co-localization in the S32T-Prdx6 lung sections as compared with wild type (Table 3). Thus, as for the A549 cells, the presence of the S32T mutation in Prdx6 appeared to inhibit its targeting to LB.

We further evaluated LB targeting by the isolation of LB from WT and S32T mice. By immunoblotting, Prdx6 was present in LB from WT mice but was essentially absent in lamellar bodies isolated from lungs of S32T-Prdx6 mice despite approximately equal Prdx6 expression in WT and mutant mouse lung homogenates (Fig. 8A). As a control, the expression of surfactant protein-A, a protein that is predominantly localized to LB and the extracellular space in lungs, was not different in LB isolated from WT and S32T-Prdx6 mice. Absence of Prdx6 protein was associated with markedly decreased aiPLA₂ activity in LB of S32T-Prdx6 mutant mice (Fig. 8B). Confirming the findings for Prdx6 expression, aiPLA₂ activity in homogenates of whole lung tissue from these mutant mice was similar to levels in WT

**TABLE 3**

Analysis for co-localization of lamellar body membrane marker (LAMP1) and Prdx6 using Manders’ overlap and Pearson’s correlation coefficients

|          | Manders | Pearson |
|----------|---------|---------|
|          | A549 cells | Lung | A549 cells | Lung |
| Wild type| 0.67    | 0.77 | 0.48 | 0.75 |
| S32T-Prdx6 | 0.44 | 0.43 | 0.19 | 0.26 |
| S32A-Prdx6 | 0.30 | ND*  | 0.02 | ND* |

* ND means not done.
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FIGURE 8. Prdx6 expression in lung LB. A, Western blot analysis for Prdx6, GAPDH, and surfactant protein-A (SPA) expression in protein samples (30 mg) from lung homogenates or lamellar bodies isolated from wild type mice or mice carrying the S32T mutation of Prdx6. B, aiPLA2 activity assayed in isolated lamellar bodies and lung tissue of WT and Prdx6-S32T mice. Values are mean ± S.E. for n = 3 independent experiments. *, p < 0.05 versus corresponding WT.

lungs (Fig. 8B). The essentially normal content of Prdx6 in the whole lung of the S32T-Prdx6 mutant mouse but its absence in LB is consistent with a defect in Prdx6 trafficking.

Serine 32 to Threonine Mutation Abolishes Prdx6 Interaction with the 14-3-3 Molecular Chaperone—Our earlier studies indicated that intracellular trafficking of Prdx6 to lysosomal organelles along the exocytotic pathway relies on its binding to a molecular chaperone, 14-3-3ε. We demonstrated that amino acid residues 31–40 of Prdx6 comprising the targeting peptide directly bind to 14-3-3ε and showed that the mutation of Ser-32 to alanine in this sequence markedly decreases interaction of the peptide and chaperone in vitro (21). To evaluate whether the S32T mutation also interferes with Prdx6 binding to 14-3-3ε, the possible interaction of the two proteins in the intact cell was studied with the Duolink in situ proximity ligation assay. A signal indicating proximity of the two proteins was detected in WT lungs but not in Prdx6-S32T mouse lungs (Fig. 9). These findings provide additional evidence that 14-3-3ε modulates Prdx6 intracellular trafficking to lysosomal organelles and provides a mechanism for the altered targeting of Prdx6-S32T mutant proteins to lamellar bodies.

Effect of Altered Prdx6 Lamellar Body Targeting of Prdx6-S32T on Lung Surfactant Phospholipid Metabolism—We reasoned that impaired trafficking of Prdx6 to LB in mice would result in a phenotype that is similar to the Prdx6 null mouse with respect to LB phospholipid metabolism. Our previous studies in Prdx6 null mice indicated that Prdx6 plays an important role in the metabolism of lung surfactant DPPC. DPPC is the major bioactive phospholipid component of lung surfactant (1, 43). After being endocytosed by alveolar epithelial cells, lung surfactant DPPC is degraded in LB by lysosome-type aiPLA2 (i.e. Prdx6).

Lyso-PC, a product of aiPLA2 activity, serves as the substrate for reacylation to regenerate DPPC by the remodeling pathway. Absence of aiPLA2 activity in the Prdx6 null mouse results in the accumulation of phospholipids in lung tissue (33). However, results for the null mouse are confounded by the absence of Prdx6 from the whole cell. Thus, the precise role of LB Prdx6 in lung PC metabolism has not as yet been demonstrated. The present model where Prdx6 is absent only in LB represents an opportunity to evaluate the specific role of LB Prdx6 in lung surfactant phospholipid metabolism.

To evaluate the role of LB Prdx6, we investigated the uptake and degradation of extracellular DPPC in isolated perfused mouse lungs. Phospholipid composition was measured in the lung tissue, lung lavage (BAL) fluid, and isolated lamellar bodies from WT and S32T mutant mouse lungs. Total phospholipid, DSPC, and PC contents in all three compartments in S32T mutant mouse lungs were significantly increased compared with WT lungs (Fig. 10, A–C). The increase in phospholipids in the whole lung as well as the LB and BALF phospholipids suggests that synthesis of phospholipids and their secretion by lamellar body exocytosis were unaffected by S32T-Prdx6 mutation. We next evaluated the recycling (uptake and metabolism) of lung phospholipids.

Uptake of [3H]DPPC by the isolated perfused lung was measured at 2 h after instillation of mixed unilamellar liposomes into alveolar spaces and was not significantly different in WT versus S32T-Prdx6 mouse lungs (Fig. 11A). The lyso-PC fraction is generated by PLα2 activity, whereas the aqueous fraction is derived from further breakdown of lyso-PC to choline and its metabolite derivatives. The unsaturated PC fraction represents PC containing unsaturated fatty acid in the sn-2 position, which is generated by the reacylation of lyso-PC. Total lung degradation of DPPC was decreased by ~50% (p < 0.05) in Prdx6-S32T
The decrease in dpm recovery was greatest in the unsaturated PC fraction, whereas the lyso-PC and aqueous fractions showed decreases of a lesser degree (Fig. 11B). We have previously shown similar changes, compared with WT, in Prdx6 null lungs and in lungs treated with a transition state-mimic inhibitor of Prdx6 PLA2 activity (17, 33). These data indicate that targeted lamellar body depletion of Prdx6 in S32T lungs affects lamellar body phospholipid degradation leading to accumulation of phospholipids in lungs and LB.

Discussion

A major goal for this study was to compare the substitution of Ala versus Thr for Ser-32 in Prdx6 to evaluate the effect on enzymatic activities of the protein. The Ser-32 residue is a component of the Prdx6 catalytic triad and is specifically required for the PLA2 activity of Prdx6 (20). This residue is conserved in mammalian (human, bovine, rat, and mouse) Prdx6 (9, 25), avian (chicken, Gallus gallus), and amphibian (frog, Xenopus tropicalis) Prdx6 (GenBank™), many species of fish Prdx6 (44), insect (Drosophila dPrx 2540 and dPrx 6005) (45), plant (cress, Arabidopsis thaliana) (GenBank™), and yeast mitochondrial thioredoxin peroxidase (45). The Drosophila, plant, and yeast proteins are 1-cysPrdx enzymes that are homologous to Prdx6. The only 1-cysPrdx enzyme that we have identified in the literature as lacking Ser in position 32 is the yeast nuclear thioredoxin peroxidase that has been observed to be the least homologous of any member of the family (45). This high degree of conservation of the Ser-32 residue is compatible with its key role in Prdx6 enzymatic function. However, to date, only the mammalian enzymes have been shown to exhibit PLA2 activity. Mutation of Ser-32 to Ala abolished Prdx6 PLA2 activity in human (this study and Ref. 25) and rat (20) Prdx6, but the substitution of Thr for Ser had no effect on enzymatic activity. We have not found any instance of the substitution of Thr for Ser-32 in a natural Prdx6/1-cysPrdx protein.

A secondary goal of this study was to evaluate the effect of targeted depletion of lamellar body PLA2 on lung phospholipid metabolism. Our earlier studies of mice either lacking or over-expressing Prdx6 have indicated that the aiPLA2 activity of this protein plays a major role in the regulation of lung surfactant phospholipid homeostasis (17, 33). Treatment of lungs with MJ33, a specific PLA2 inhibitor, markedly diminished the degradation of surfactant DPPC after its internalization by lung type 2 alveolar epithelial cells in situ (17, 33). However, it was not possible using these latter models to specifically investigate the role of lamellar body PLA2 in lung surfactant phospholipid turnover because the genetic manipulations or use of the inhibitor resulted in global inhibition of PLA2 activity. The phenotype of the Prdx6-S32T knock-in mouse with Prdx6 depletion only from LBs of lung epithelial cells gave us the opportunity to...
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study the specific role of lamellar body aiPLA2 activity in the degradation and remodeling of lung surfactant PC.

Analysis of protein expression in lung tissue and lamellar bodies isolated from WT and S32T mice confirmed that Prdx6 was equally expressed in WT and mutant mouse lungs but was essentially absent in lamellar bodies isolated from mutant lung homogenates. The S32T Prdx6 mutant lungs showed significantly diminished degradation of DPPC and the accumulation of phospholipids. Although this study evaluated a single time point in terms of mouse age, we have shown previously that the lungs of Prdx6 null mice accumulate DPPC, PC, and total phospholipids at a linear rate between the ages of 4 and 48 weeks (17). The lipid composition of wild type lungs (normalized to body weight) of a similar age range was constant. The values obtained in this study of mice with absent LB Prdx6 were nearly identical to values that were obtained 10 years ago for Prdx6 null mice of a similar age with absent Prdx6 in all lung compartments (17). Whereas the endoplasmic reticulum is the primary site for synthesis of lung surfactant phospholipids by the de novo pathway, the LB are the site for phospholipid (surfactant) “storage” (1, 5). Our previous studies have shown that LB are also a site for degradation or remodeling of phospholipids that have been endocytosed from the alveolar space (recycled surfactant) (4, 15–17, 33). The results of this study confirm that phospholipid degradation and remodeling in LB requires Prdx6 and that LB Prdx6 indeed has an important role in the normal turnover of lung phospholipids.

Our earlier studies indicated that Prdx6 localization to LB may rely on the protein binding to 14-3-3ε, a molecular chaperone that is known to facilitate transport of signaling molecules along the secretory pathway (21). Serine 32 to alanine mutation in the Prdx6(31–40) amino acid lysosomal targeting motif diminished the interaction of Prdx6 with 14-3-3ε in vitro and in cells and abolished lysosomal localization of Prdx6 (21). This mutation also resulted in the inactivation of the aiPLA2 activity of Prdx6. In this study, the serine to threonine substitution in the Prdx6-S32T knock-in model showed no effect on protein enzymatic activity but resulted in the loss of protein interaction with the 14-3-3ε chaperone molecule, thus resulting in a lack of targeting to LB and presumably to other lysosome-like organelles.

Throneine, like serine, is a small weakly polar amino acid, and substitution of one for the other generally has little effect on enzymatic activity (47). Thus, Thr can substitute for Ser in a range of enzymatic activities, including the PLA2 activity of Prdx6. Many protein kinases as well as phosphatases catalyze phosphorylation/dephosphorylation of either serine or threonine sites in proteins with generally similar activities toward either amino acid residue. However, there are some reports that have shown a change in enzymatic activity with serine to threonine mutation. For example, activity of alcohol dehydrogenase from Thermoanaerobacter ethanolicus, using 2-propanol as a substrate, was increased in the S39T mutant without a significant effect on NADPH binding; it was proposed that the serine to threonine substitution permitted a change in the steric environment of the active site without disrupting the essential proton relay system in which the Ser-39 hydroxyl group participates (48). As another example, the T1S mutation of the 26S proteasome decreases proteolytic activity by an order of magnitude (53). Also, changing Ser-65 to threonine in the green fluorescent protein (GFP) chromophore region was reported to stabilize the hydrogen bonding network in the chromophore, resulting in the enhancement of the fluorescent signal (49). In the present results, decreased binding of S32T-Prdx6 to an important chaperone protein, 14-3-3ε, resulted in altered transport to lung lamellar bodies, with subsequent impairment of LB PLA2 activity and altered surfactant phospholipid metabolism.

Changes in phospholipid content represent a hallmark for lysosomal storage disorders that result from the deficiency of LB hydrolases, and increased lung phospholipid content of 4–6-fold has been reported in lungs of patients affected by sialidosis and Gaucher and Sandhoff diseases (51). A similar increase in lung phospholipids has been found in mouse models of these diseases as, for example, the β-hexosaminidase-deficient mouse (50). We have recently evaluated pearl mice that are a model of the Hermansky-Pudlak syndrome, a disease of protein trafficking and organelar dysfunction (52). These lungs showed impaired Prdx6 targeting to LB and lung phospholipidosis (54). Understanding the mechanisms that regulate localization of lysosomal cargo proteins may lead to the development of novel therapies for some lysosomal storage diseases.

In conclusion, this study demonstrates that Thr can substitute for Ser at the active site for the PLA2 activity of Prdx6. The study also presents a mouse model of targeted depletion of Prdx6 in the lung lamellar bodies and confirms the role of these organelles in lung surfactant phospholipid metabolism. Finally, we confirm that Prdx6 subcellular targeting to lysosome-related organelles relies on its interaction with the chaperone molecule 14-3-3ε, thereby establishing the importance of the Ser-32 residue of Prdx6 for protein-protein interactions and subsequent organelar localization of Prdx6.

Author Contributions—E. M. S. participated in project design, performed histology, PAGE, and peroxidase and Duolink assays, generated constructs for protein expression in cells, and wrote the first draft of the manuscript; C. D. performed phospholipid metabolism studies and PLA2 assays; S. Z. generated lentivirus constructs; J. Q. T. prepared lung sections for histologic analysis; L. G. prepared the codon-optimized plasmid and generated recombinant Prdx6; T. R. generated the constructs for the knock-in mice; S. I. F. participated in the conceptualization of the project and supervised the generation of mutant constructs; A. B. F. participated in project conceptualization and design and edited the manuscript.

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21. Manevich, Y., Feinstein, S. I., Zhou, S., and Fisher, A. B. (1996) Intracellular processing of surfactant lipids in the lung. *Annu. Rev. Physiol.* 57, 789–802

22. Goss, V., Hunt, A. N., and Postle, A. D. (2013) Regulation of lung surfactant metabolism and binding. *Biochim. Biophys. Acta* 1831, 448–458

23. Whitsett, J. A., Wett, S. E., and Weaver, T. E. (2015) Diseases of pulmonary surfactant homeostasis. *Annu. Rev. Pathol.* 10, 371–393

24. Whitsett, J. A., Wett, S. E., and Weaver, T. E. (2015) Diseases of pulmonary surfactant homeostasis. *Annu. Rev. Pathol.* 10, 371–393

25. Schmidt, R., Meier, U., Markart, P., Grimminger, F., Velcovsky, H. G., Morr, H., Seeger, W., and Günther, A. (2002) Altered fatty acid composition of lung surfactant phospholipids in intestinal lung disease. *Am. J. Physiol. Lung Cell Mol. Physiol.* 283, L1079–L1085

26. Kim, T. S., Dodia, C., Chen, X., Hennigan, B. B., Jain, M., Feinstein, S. I., and Fisher, A. B. (1998) Cloning and expression of rat lung acidic Ca$^{2+}$-independent PLA2 and its organ distribution. *Am. J. Physiol.* 274, L750–L761

27. Li, H., Benipal, B., Zhou, S., Dodia, C., Chatterjee, S., Tao, J. Q., Sorokina, E. M., Raabe, T., Feinstein, S. I., and Fisher, A. B. (2015) Critical role of peroxiredoxin 6 in protection against oxidative stress in the mouse lung. *Antioxid. Redox Signal.* 23, C723–C742

28. Dong, Q. G., Bernasconi, S., Lostaglio, S., De Calmanovici, R. W., Martin-Padura, I., Breviario, F., Garlanda, C., Ramponi, S., Mantovani, A., and Vecchi, A. (1997) A general strategy for isolation of endothelial cells from murine tissues. Characterization of two endothelial cell lines from the murine lung and subcutaneous sponge implants. *Arterioscler. Thromb. Vasc. Biol.* 17, 1599–1604

29. Wei, Z., Al-Mehdi, A. B., and Fisher, A. B. (2001) Signaling pathway for nitric oxide generation with simulated ischemia in flow-adapted endothelial cells. *Am. J. Physiol. Heart Circ. Physiol.* 281, H2226–H2232

30. Benipal, B., Feinstein, S. I., Chatterjee, S., Dodia, C., and Fisher, A. B. (2015) Inhibition of the phospholipase A activity of peroxiredoxin 6 prevents lung damage with exposure to hyperoxia. *Redox Biol.* 4, 321–327

31. Omdorf, R. L., Hong, N., Yu, K., Feinstein, S. I., Zern, B. J., Fisher, A. B., Muzykantov, V. R., and Chatterjee, S. (2014) NOX2 in lung inflammation: quantum dot based in situ imaging of NOX2-mediated expression of vascular cell adhesion molecule-1. *Am. J. Physiol. Lung Cell Mol. Physiol.* 306, L260–L268

32. Fisher, A. B., Dodia, C., Ruckert, P., Tao, J. Q., and Bates, S. R. (2010) Pathway to lamellar bodies for surfactant protein A. *Am. J. Physiol. Lung Cell Mol. Physiol.* 299, L51–L58

33. Fisher, A. B., and Dodia, C. (2001) Lysosomal-type PLA2 and turnover of alveolar phospholipid. *Am. J. Physiol. Lung Cell Mol. Physiol.* 280, L1408–L1415

34. Omdorf, R. L., Hong, N., Yu, K., Feinstein, S. I., Zern, B. J., Fisher, A. B., Muzykantov, V. R., and Chatterjee, S. (2014) NOX2 in lung inflammation: quantum dot based in situ imaging of NOX2-mediated expression of vascular cell adhesion molecule-1. *Am. J. Physiol. Lung Cell Mol. Physiol.* 306, L260–L268

35. Fisher, A. B., Dodia, C., Ruckert, P., Tao, J. Q., and Bates, S. R. (2010) Pathway to lamellar bodies for surfactant protein A. *Am. J. Physiol. Lung Cell Mol. Physiol.* 299, L51–L58

36. Fisher, A. B., and Dodia, C. (2001) Lysosomal-type PLA2 and turnover of alveolar phospholipid. *Am. J. Physiol. Lung Cell Mol. Physiol.* 280, L1408–L1415

37. Fisher, A. B., and Dodia, C. (2001) Lysosomal-type PLA2 and turnover of alveolar phospholipid. *Am. J. Physiol. Lung Cell Mol. Physiol.* 280, L1408–L1415

38. Goerke, J. (1998) Pulmonary surfactant: functions and molecular composition. *Biochim. Biophys. Acta* 1408, 79–89

39. Bligh, E. G., and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37, 911–917

40. Dunn, K. W., Kamocka, M. M., and McDonald, J. H. (2011) A practical guide to evaluating colocalization in biological microscopy. *Am. J. Physiol. Cell Physiol.* 300, C723–C742

41. Fisher, A. B., Arad, I., Dodia, C., Chatterjee, S., and Feinstein, S. I. (1991) cAMP increases synthesis of surfactant-associated protein A by perfused rat lung. *Am. J. Physiol.* 260, L226–L233

42. Liu, G., Feinstein, S. I., Wang, Y., Dodia, C., Fisher, D., Yu, K., Ho, Y. S., and Fisher, A. B. (2010) Comparison of glutathione peroxidase 1 and peroxiredoxin 6 in protection against oxidative stress in the mouse lung. *Free Radic. Biol. Med.* 49, 1172–1181

43. Wang, Y., Feinstein, S. I., and Fisher, A. B. (2008) Peroxiredoxin 6 as an antioxidant enzyme: protection of lung alveolar epithelial type II cells from oxidative stress. *Nat. Med.* 14, 1750–1757

References

1. Schmitz, G., and Müller, G. (1991) Structure and function of lamellar bodies, lipid-protein complexes involved in storage and secretion of cellular lipids. *J. Lipid Res.* 32, 1539–1570

2. Marks, M. S., Heijnen, H. F., and Raposo, G. (2013) Lysosome-related organelles: unusual compartments become mainstream. *Curr. Opin. Cell Biol.* 25, 495–505

3. Chander, A., Johnson, R. G., Reichert, J., and Fisher, A. B. (1986) Lamellar bodies maintain an acidic internal pH. *J. Biol. Chem.* 261, 6126–6131

4. Fisher, A. B., and Chander, A. (1985) Intracellular processing of surfactant lipids in the lung. *Annu. Rev. Physiol.* 47, 789–802

5. Goss, V., Hunt, A. N., and Postle, A. D. (2013) Regulation of lung surfactant phospholipid synthesis and metabolism. *Biochim. Biophys. Acta* 1831, 448–458

6. Whitsett, J. A., Wett, S. E., and Weaver, T. E. (2015) Diseases of pulmonary surfactant homeostasis. *Annu. Rev. Pathol.* 10, 371–393

7. Veldhuizen, R., Nag, K., Orgeig, S., and Possmayer, F. (1998) The role of lipids in pulmonary surfactant. *Biochim. Biophys. Acta* 1408, 90–108

8. Schmidt, R., Meier, U., Markart, P., Grimmer, F., Velkovsky, H. G., Morr, H., Seeger, W., and Günther, A. (2002) Altered fatty acid composition of lung surfactant phospholipids in intestinal lung disease. *Am. J. Physiol. Lung Cell Mol. Physiol.* 283, L1079–L1085

9. Kim, T. S., Dodia, C., Chen, X., Hennigan, B. B., Jain, M., Feinstein, S. I., and Fisher, A. B. (1998) Cloning and expression of rat lung acidic Ca$^{2+}$-independent PLA2 and its organ distribution. *Am. J. Physiol.* 274, L750–L761
Peroxiredoxin 6 Lamellar Body Targeting

H2O2-induced oxidative stress. J. Cell. Biochem. 104, 1274–1285
43. Widdicombe, J. G. (1987) Role of lipids in airway function. Eur. J. Respir. Dis. 153, 197–204
44. Tolomeo, A. M., Carraro, A., Bakiu, R., Toppo, S., Place, S. P., Ferro, D., and Santovito, G. (2016) Peroxiredoxin 6 from the Antarctic emerald rockcod: molecular characterization of its response to warming. J. Comp. Physiol. B 186, 59–71
45. Rhee, S. G., Kang, S. W., Chang, T. S., Jeong, W., and Kim, K. (2001) Peroxiredoxin, a novel family of peroxidases. IUBMB Life 52, 35–41
46. Wang, Y., Phelan, S. A., Manevich, Y., Feinstein, S. I., and Fisher, A. B. (2006) Transgenic mice overexpressing peroxiredoxin 6 show increased resistance to lung injury in hyperoxia. Am. J. Respir. Cell Mol. Biol. 34, 481–486
47. Betts, M. J. and Russell, R. B. (2003) in Bioinformatics for Geneticists (Barnes, M. R., and Gray, I. C., eds) John Wiley & Sons, Ltd., New York, 10.1002/0470867302.ch14
48. Tripp, A. E., Burdette, D. S., Zeikus, J. G., and Phillips, R. S. (1998) Mutation of Serine-39 to threonine in thermostable secondary alcohol dehydrogenase from Thermobacter ethanolicus changes enantiospecificity. J. Am. Chem. Soc. 120, 5137–5141
49. Tsien, R. Y. (2010) Nobel lecture: constructing and exploiting the fluorescent protein paintbox. Integr. Biol. 2, 77–93
50. Buccoliero, R., Palmeri, S., Ciarleglio, G., Collodoro, A., De Santi, M. M., and Federico, A. (2007) Increased lung surfactant phosphatidylcholine in patients affected by lysosomal storage diseases. J. Inherit. Metab. Dis. 30, 983–983
51. Buccoliero, R., Ginzburg, L., and Futerman, A. H. (2004) Elevation of lung surfactant phosphatidylcholine in mouse models of Sandhoff and of Niemann-Pick A disease. J. Inherit. Metab. Dis. 27, 641–648
52. Feng, L., Seymour, A. B., Jiang, S., To, A., Peden, A. A., Novak, E. K., Zhen, L., Rusiniak, M. E., Eicher, E. M., Robinson, M. S., Gorin, M. B., and Swank, R. T. (1999) The β3A subunit gene (Ap3b1) of the AP-3 adaptor complex is altered in the mouse hypopigmentation mutant pearl, a model for Hermansky-Pudlak syndrome and night blindness. Hum. Mol. Genet. 8, 323–330
53. Kisselev, A. F., Songyang, Z., and Goldberg, A. L. (2000) Why does threonine, and not serine, function as the active site nucleophile in proteasomes? J. Biol. Chem. 275, 14831–14837
54. Guttentag, S. H., Zhang, P., Wang, P., Sorokina, E. M., Dodia, C., and Fisher, A. B. (2012) Peroxiredoxin 6 is an AP-3-dependent cargo protein trafficked to lamellar bodies. Am. J. Respir. Crit. Care Med. 185, A1984