A novel lysophosphatidylcholine acyl transferase activity is expressed by peroxiredoxin 6

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Abstract The phospholipase A_2 (PLA_2) activity of peroxiredoxin (Prdx)6 has important physiological roles in the synthesis of lung surfactant and in the repair of peroxidized cell membranes. These functions require the activity of a lysophospholipid acyl transferase as a critical component of the phospholipid remodeling pathway. We now describe a lysophosphatidylcholine acyl transferase (LPCAT) activity for Prdx6 that showed a strong preference for lysophosphatidylcholine (LPC) as the head group and for palmitoyl CoA in the acylation reaction. The calculated kinetic constants for acylation were K_m 18 µM and V_{max} 30 nmol/min/mg protein; the V_{max} was increased 25-fold by phosphorylation of the protein while K_m was unchanged. Study of recombinant protein in vitro and in mouse pulmonary microvascular endothelial cells infected with a lentiviral vector construct indicated that amino acid D31 is crucial for LPCAT activity. A linear incorporation of labeled fatty acyl CoA into dipalmitoyl phosphatidylcholine (PC) indicated that LPC generated by Prdx6 PLA_2 activity remained bound to the enzyme for the reacylation reaction. Prdx6 is the first LPCAT enzyme with demonstrated cytoplasmic localization.ᴶᴸᴿ Thus, Prdx6 is a complete enzyme comprising both PLA_2 and LPCAT activities for the remodeling pathway of PC synthesis or for repair of membrane lipid peroxidation.—Fisher, A. B., C. Dodia, E. M. Sorokina, H. Li, S. Zhou, T. Raabe, and S. I. Feinstein. **A novel lysophosphatidylcholine acyl transferase activity is expressed by peroxiredoxin 6.** *J. Lipid Res.* 2016. **57: 587–596.**

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Peroxiredoxin (Prdx)6 is a 1-cys member of the Prdx family that has the unique combination of activities for both reduction of phospholipid hydroperoxides (as well as short chain hydroperoxides) using GSH as an electron donor and for hydrolysis of phospholipids; i.e., it exhibits both GSH peroxidase (GPx) and phospholipase A_2 (PLA_2) activities (1). These activities require the binding of Prdx6 to phospholipids in a specific orientation in relation to cys47, the active site for GPx activity, and to S32-H26-D140, the catalytic triad for PLA_2 activity (2, 3). The GPx activity of Prdx6 plays a key role in the antioxidant defense of the lung, as well as other organs (4–8), and has been shown recently to participate in the repair of peroxidized cell membranes by reduction of phospholipid hydroperoxides (3, 9). The PLA_2 activity of Prdx6 also participates in the repair of peroxidized cell membranes by liberating the *sn*-2 oxidized fatty acid to generate lysophosphatidylcholine (LPC) (9, 10). Additionally, the PLA_2 activity participates in the turnover of lung surfactant phospholipids with a major function in phospholipid remodeling to generate the dipalmitoylphosphatidylcholine (DPPC) that is the surface-active lipid component of the lung surfactant (11–15). Both the repair of peroxidized cell membrane phospholipids and a pathway for DPPC synthesis require the combined activity of PLA_2 followed by a LPC acyl transferase (LPCAT) activity in order to either regenerate a reduced membrane phospholipid or to remodel phosphatidylcholine (PC) to produce DPPC. This pathway that combines PLA_2 activity followed by LPCAT activity has been designated as the remodeling pathway for PC synthesis (Lands cycle) (16).

These metabolic functions of PLA_2 that are associated with phospholipid turnover and membrane repair are clearly compartmentalized within cells. While DPPC synthesis via the de novo (Kennedy) pathway occurs in the

endoplasmic reticulum (ER) (17), the hydrolysis of PC to generate LPC as the substrate for reacylation in the remodeling pathway for lung surfactant DPPC synthesis has been localized to lysosomal-related organelles [LROs, known as lamellar bodies (LBs) in the lung epithelial cells] (15, 18). Prdx6 protein is relatively enriched in the LBs (11, 19, 20) and inhibition of its PLA$_2$ activity decreases flux through the phospholipid remodeling pathway (11, 13–15, 21). In contrast, the remodeling pathway for the repair of peroxidized cell membranes presumably occurs at the cytoplasmic face of the affected cell membrane. Although Prdx6 binds to phospholipid substrate at acidic pH, the nonphosphorylated protein does not bind to phospholipids at cytoplasmic pH; however, it does bind at pH 7 to peroxidized phospholipids (3, 9, 22). Thus, the physiological function of the PLA$_2$ activity of nonphosphorylated Prdx6 occurs either in lysosomes (or LROs) that have an acidic internal pH or at the interface of the cytoplasm and the oxidized cell membrane. Phosphorylation of the enzyme results in a significant increase in membrane binding at pH 7, as well as at pH 4 (22, 23).

Recognizing the disparate sites for these physiological functions raises a question concerning the mechanism for coupling of the PLA$_2$ activity to the subsequent LPCAT activity. All LPCAT activities that have been described to date are localized primarily to the ER (24). In the case of lung surfactant, this has led to speculation regarding cooperativity between the LBs and ER in the synthesis of DPPC (18, 25). The source of LPCAT activity for the repair (remodeling) of peroxidized cell membrane phospholipids is also not clear (9). Thus, we investigated whether Prdx6 that is present in LBs and cytoplasm might express LPCAT activity.

MATERIALS AND METHODS

Animals

All procedures involving mice were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania and use of mice conformed to the Public Health Service policy on the humane care and use of laboratory animals. Wild-type C57Bl/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME). Prdx6-null and mutant mice were bred in the animal care facilities of the University of Pennsylvania. All mutations are expressed on the C57Bl/6J background. The generation of Prdx6-null mice and "knock-in" mice with the C47S and D140A mutations of Prdx6 and their genotyping by PCR has been described previously (9, 26, 27).

For lung isolation, mice were anesthetized with intraperitoneal ketamine:xylazine:acepromazine (100:15:2 mg/kg body weight); their lungs were cleared of blood, isolated from the thorax, and studied as an isolated perfused lung preparation as described previously (10, 26).

H26A Prdx6 mouse

For the present study, we also generated H26A Prdx6 knock-in mice using constructs designed by the Gene Targeting Core and Laboratory and chimeric mice that were generated by the Transgenic and Chimeric Mouse Facility of the University of Pennsylvania. To retrieve the part of the *prdx6* gene to be mutated for generation of the H26A mutation, short homologous arms were amplified from a *prdx6* gene containing BAC clone; 300 bp sequences were amplified by PCR to generate linear fragments flanked by either *Not*I/*Hin*dIII or *Hin*dIII/*Spe*I, respectively. These were then coligated into the pL253 retrieval vector (National Cancer Institute at Frederick recombineering website; http://redrecombineering.ncifcrf.gov) using the *Not*I and *Spe*I sites in the mutiple cloning site (MCS) region. The resulting construct was linearized using *Hin*dIII and transfected into *Escherichia coli* SW102 containing heat shock inducible lambda Red recombination proteins and the *prdx6* BAC clone. Following treatment at 42°C for 15 min and ampicillin selection, 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. This fragment served as the basic backbone for the H26A construct (**Fig. 1A**).

To generate the H26A mutant allele, a mini vector was generated first. An ∼300 bp long genomic *prdx6* fragment located ∼150 nt 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 Cancer Institute at Frederick, recombineering website). The two PCR fragments were ligated into PL451 by two subsequent ligation and cloning cycles. This pL451 derivative was mutated at codon 26 in exon 1 by site-directed mutagenesis (H26A; CAC converted to GCC) to derive the final mutagenic mini targeting vector. This mini vector, which also contained a pgk EM7 neo polyA cassette flanked by FLP recombinase target sites, was linearized, transfected, and recombined into the above ∼12 kB prxd6 vector backbone by heat induction of the lambda Red recombination enzymes. Generation of the final targeting vector plasmid with the H26A mutation was performed in bacteria by selection for the neomycin resistance gene 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. Positive ES clones showed homologous recombination using Southern blotting (Fig. 1B); these clones were karyotyped, the H26A mutation verified by genomic sequencing, and finally used for blastocyst injection into CD-1/BALB/c mice.

Chimeric H26A Prdx6 mice were bred to C57Bl/6J wild-type mice and the resulting heterozygotic mice were bred to homozygosity that was confirmed by PCR (Fig. 1C) using previously published protocols (9). PCR (35 cycles) was performed with denaturation at 95°C (30 s), annealing at 71°C (15 s), and elongation at 72°C (30 s). For wild-type, the specific oligonucleotide primer for the forward reaction for wild-type was: 5′-CATCGGCC-GCATCCGCTTC**CA**-3′. For H26A, the oligonucleotide primer for the forward reaction was: 5′-CATCGGCCGCATCCGCTTC**GC**-3′. Bolded letters indicate the nucleotides that differ between the wild-type and the mutant sequences. The reverse primer for both reactions was: 5′-CAGACAACAAACCGTCCTCGGCAAG-3′. The PCR products, 317 bp in size, were subjected to electrophoresis on a 1.5% agarose gel.

Proteins, endothelial cells, and LBs

Constructs for generation of recombinant human wild-type Prdx6 (hPrdx6) protein using codon-optimized primers and recombinant rat Prdx6 (rPrdx6) protein and the subsequent protein purification using ion-exchange chromatography have been described previously (2, 28–30). Prdx6 was phosphorylated at amino acid T177 by incubation with the MAPK, Erk 2, in the presence of Mg$^+$-ATP, as described previously (22, 23).

Endothelial cells from wild-type Prdx6-null and Prdx6-mutant mice were isolated from lungs using enzymatic digestion followed
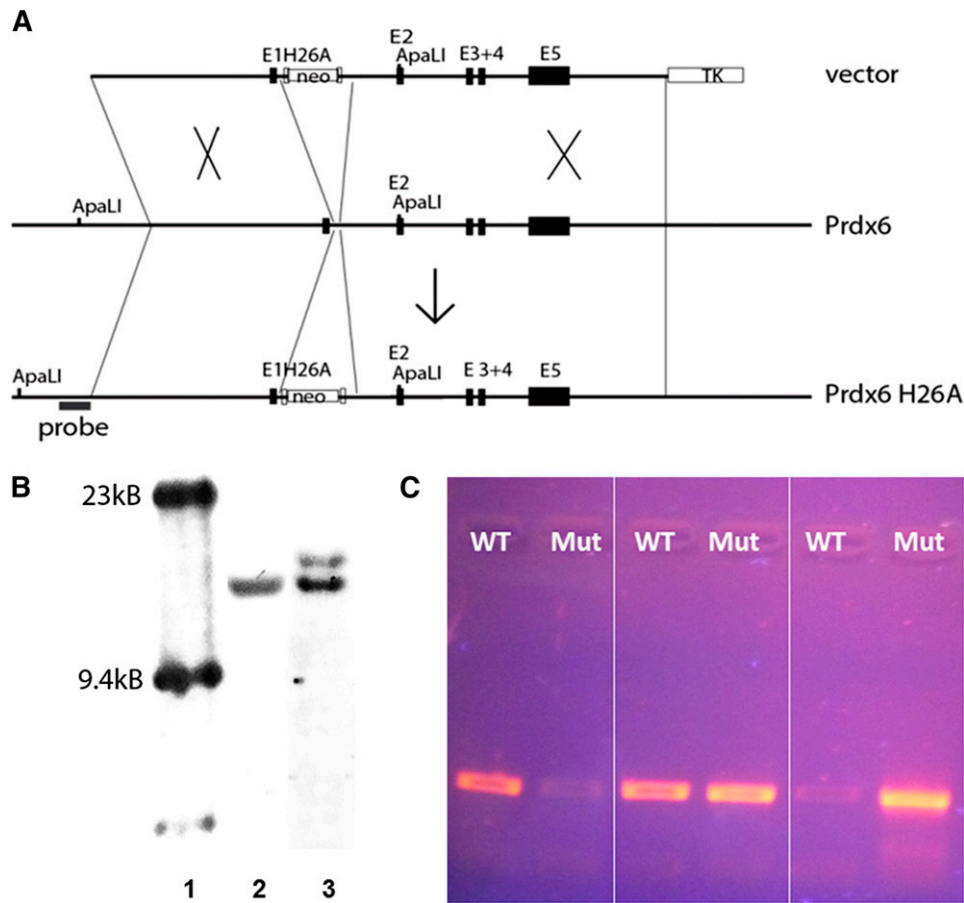
Fig. 1. Schematic for generation of Prdx6 H26A mutant mice. A: A targeting vector was constructed with an FLP recombinase target site-flanked neo cassette 290 bp downstream of exon 1 and electroporated into C57Bl6 ES cells. Neo-positive ES clones were screened by Southern blotting using a probe located just upstream of the 5′ end of the vector. B: The probe that was used for Southern blotting of ApaLI cut genomic DNA yielded the expected 14.9 kB band; positive ES clones yielded the wild-type band and also a 16.7 kB mutant band due to insertion of the neo cassette. Lane 1, standards; lane 2, wild-type; lane 3, H26A mutant. C: Mice were genotyped using allele-specific PCR. The wild-type specific primers identify the wild-type allele and the mutant primers identify the H26A mutation. The presence of a band at 317 bp with the specific primer indicates the presence of that allele. The primers (wild-type or mutant) are indicated above the gels. Left panel: wild-type mouse; middle panel: heterozygous mouse; right panel: homozygous H26A Prdx6 mouse.

by differential adherence (10, 31); cells were maintained in primary culture and were used at passage 7. The lentiviral vector constructs and procedures for infection of endothelial cells to generate D140A Prdx6 and C47S Prdx6 have been described previously (9). We also designed lentiviral vector constructs for expression of human H26A and D31A Prdx6. The forward primer for H26A Prdx6 was: 5′-GTCGGCCGCATCCGTTTC*GCC*GACTTT-CTGGGAGACTC-3′, and the reverse primer was: 5′-GAGTCT-CCCAGAAAGTC*GGC*GAAACGGATGCGGCCGAC-3′. For D31A Prdx6, the forward primer was: 5′-GTTCCACGACTTTCTGGG-A*GCC*TCATGGGCATTCTCTTC-3′, and the reverse primer was: 5′-GAAGAGAATGCCCCATGA*GGC*TCCCAGAAAGTCGTGGAAC-3′. The mutated codons are indicated in bold italic.

LBs were isolated by homogenization of mouse lungs that had been cleared of blood followed by density gradient centrifugation; this method produces a relatively pure population of largely intact LBs with a phospholipid to protein ratio of approximately 10 (15, 32).

Enzyme assays

PLA$_2$ activity was assayed using unilamellar liposomes (0.5 DPPC:0.25 egg PC:0.15 cholesterol:0.1 phosphatidylglycerol; mole fraction), reflecting the approximate composition of pulmonary surfactant, that were radiolabeled with ^{3}H-palmitate in the *sn*-2 position of DPPC (14). This assay measures the release of ^{3}H-palmitate as an index of PLA$_2$ activity. LPCAT activity was generally measured by the incorporation of ^{14}C-palmitoyl CoA into PC in the presence of LPC as an acyl acceptor. The standard assay utilized 50 μM [1-^{14}C]palmitoyl CoA and 200 μM LPC in pH 7 (100 mM Tris-HCl, 1 mM EGTA) or pH 4 (40 mM Na acetate, 5 mM EDTA) buffer at 30°C. The sources of enzymatic activity were recombinant protein or lung, cell, or LB homogenate. The reaction was stopped after 1 h incubation by adding chloroform:methanol (1:2). Lipids were separated by thin-layer chromatograpy using chloroform:methanol:ammonia:water (57:38:2.5:2.5, by volume percent) as solvent system. The lipid bands were visualized by exposure to iodine vapor and individual lipids were identified by their relative migration compared with standards applied to the same plate. The PC band was scraped and radioactivity was measured by scintillation counting. For determination of head group specificity, the LPCAT assay utilized *sn*-1-^{14}C-palmitoyl LPC as the acceptor in the presence of various unlabeled fatty acyl CoA substrates. For determination of coupling between the two reactions (PLA$_2$, LPCAT), protein was

incubated with nonlabeled liposomes in the presence of [1-^{14}C] palmitoyl CoA and its incorporation into PC was measured. Protein content was measured by the Coomassie blue reaction using bovine γ-globulin as standard (Bio-Rad, Richmond, VA).
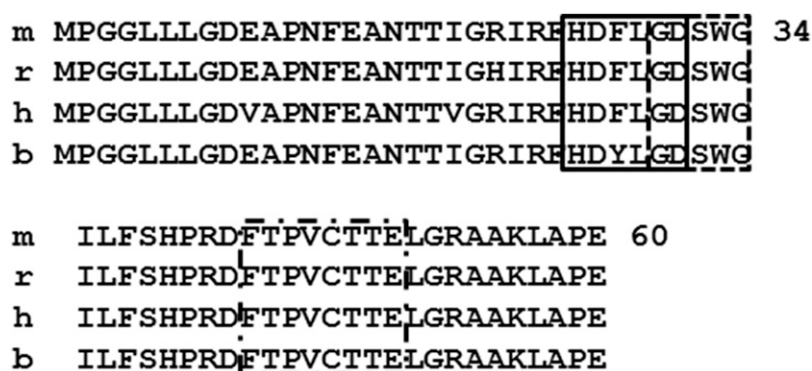
RESULTS

As a first step, we inspected the deduced Prdx6 amino acid sequences of four mammalian species (mouse, rat, human, and bovine) that are present in the Protein Data Bank for the presence of the characteristic motifs of LPCAT. The Prdx6 sequences for these four species show approximately 90% amino acid identity (33, 34). The NH$_2$-terminal 60 amino acids (out of 225 total) are shown in **Fig. 2**. A consensus lipase domain (GxSxG) that is widely expressed in lipases is seen at amino acids 30-34 (35); this sequence has an important role in the binding of phospholipids to Prdx6. S32 along with H26 and D140 represent the catalytic triad for PLA$_2$ activity (2). An eight amino acid sequence centered on C47 (FTPVCxTE) represents a consensus Prdx6 peroxidase sequence (36). A putative and conserved LPCAT motif (HxxxxD) is seen at positions 26 to 32 (Fig. 2). This putative LPCAT motif overlaps with the lipase motif and the two motifs share amino acids G30 and D31 (Fig. 2). Neither the lipase nor the LPCAT motifs are found in the other mammalian Prdxs (Prdxs 1–5) (36), and these other Prdx proteins have not been shown to bind or metabolize phospholipids.

LPCAT activity of recombinant hPrdx6 was measured by incubation of LPC with palmitoyl CoA. There was a linear increase with time in the acylation of LPC with incubation at either pH 4 or pH 7 (**Fig. 3A**); the calculated activity was nearly 3-fold greater at acidic pH (**Table 1**). Phosphorylated hPrdx6 also showed a linear rate of incorporation of palmitoyl CoA into PC during 2 h incubation and the rate of palmitoyl CoA incorporation by the phosphorylated protein was not different at pH 4 and 7 (Fig. 3B). The calculated LPCAT activity of phosphosphorylated Prdx6 was 10-fold greater at pH 4 and 27-fold greater at pH 7 than the

activity of the nonphosphorylated protein (Table 1). Results for LPCAT activity of rPrdx6 were nearly identical to those for the human protein (Table 1; Fig. 3A, B). For subsequent studies, we focused on LPCAT activity of hPrdx6 at pH 4 (reflecting the pH of lysosomes and LROs) and phosphorylated Prdx6 at pH 7 (reflecting the cytoplasmic pH, the expected subcellular site for phosphorylation of the enzyme).

We evaluated the kinetic constants for LPCAT activity by varying the palmitoyl CoA concentration. Double reciprocal plots of both the nonphosphorylated and phosphorylated proteins were linear (**Fig. 4A, B**) and indicated similar K_m values but, as expected, a significantly greater value for the V_{max} for the phosphorylated protein (**Table 2**). For comparison, Table 2 also shows kinetic constants for PLA$_2$ activity of Prdx6 at pH 4 and phosphorylated Prdx6 at pH 7 that have been published previously (37, 38). The K_m as well as V_{max} values for the PLA$_2$ activity of Prdx6 and phosphorylated Prdx6 were both greater than the corresponding kinetic constants for LPCAT activity (Table 2).

We evaluated the head group specificity for the activity of Prdx6 by substituting lysophosphatidylethanolamine, lysophosphatidylglycerol, lysophosphatidylinositol, or lysophosphatidylserine for LPC in the assay. Activity was markedly reduced by 97% or more with each of the lysophospholipid substrates as compared with LPC (**Table 3**). Thus, the acyl transferase activity of Prdx6 appears to be relatively specific for LPC. We have found previously that the enzyme also has preference for PC in its PLA$_2$ activity, with about 50% reduction when phosphatidylethanolamine was substituted for PC and greater reduction with the other head groups (19, 33). We evaluated the fatty acyl specificity for the LPCAT transferase activity by substituting acetyl CoA, stearoyl CoA, oleoyl CoA, or arachidonoyl CoA for palmitoyl CoA in the assay. Each of these as the substrate for acylation demonstrated only a low level of activity that, compared with palmitoyl CoA, was 80% less for assay with Prdx6 and >95% less in assay with phosphorylated Prdx6 (**Table 4**). The combined results in Tables 3 and 4

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m  MPGGLLLGDEAPNFEANTTIGRIRF HDFLGD SWG  34
r  MPGGLLLGDEAPNFEANTTIGHIRF HDFLGD SWG
h  MPGGLLLGDVAPNFEANTTVGRIRF HDFLGD SWG
b  MPGGLLLGDEAPNFEANTTIGRIRF HDYLGD SWG

m  ILFSHPRD FTPVCTTE LGRAAKLAPE  60
r  ILFSHPRD FTPVCTTE LGRAAKLAPE
h  ILFSHPRD FTPVCTTE LGRAAKLAPE
b  ILFSHPRD FTPVCTTE LGRAAKLAPE
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Fig. 2. Deduced amino acid sequence for Prdx6 indicating motifs associated with enzymatic activities. The N-terminal amino acid sequence (amino acids 1-60) is shown for mouse (m), rat (r), human (h), and bovine (b) Prdx6. The sequence corresponding to the dashed box indicates the lipase motif (GxSxG) that is a common feature of enzymes expressing lipase-associated activity. The sequence corresponding to the consensus Prdx motif (FTPVCxTE) is shown in the dash-dot box. The motif associated with acyl transferase activity HxxxxD is shown in the solid box. For all motifs, x indicates a nonconserved amino acid.
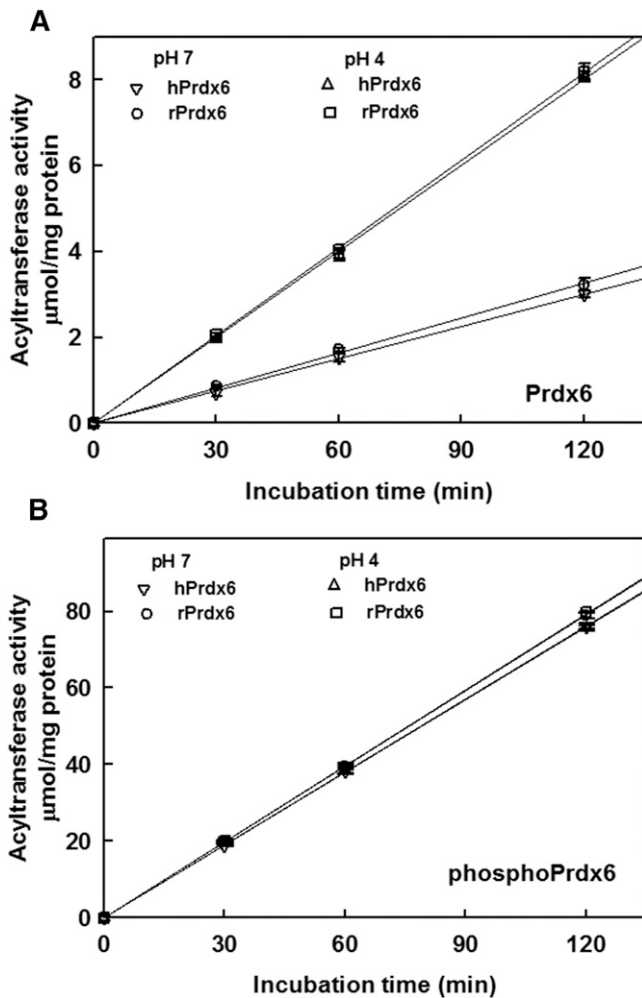
Fig. 3. LPCAT activity of recombinant hPrdx6. The assay measured incorporation of ^{14}C-palmitoyl CoA into PC in the presence of LPC as a function of time between 30 and 120 min at both pH 4 and pH 7. A: Recombinant hPrdx6 and rPrdx6 protein. B: Recombinant hPrdx6 and rPrdx6 that was phosphorylated (phopho-Prdx6). Plotted values are the mean ± SE for n = 3 independent experiments. The SE where not visible is within the width of the symbol.

indicate that Prdx6 essentially produces PC with palmitate in the sn-2 position.

We evaluated several compounds for their potential to inhibit LPCAT activity. MJ33 is a potent inhibitor of Prdx6 PLA$_2$ activity based on its structure as an analog of the substrate transition state (21). This agent inhibited PLA$_2$ activity of Prdx6 in the present study, but there was no inhibition

of LPCAT activity (data not shown). We tested the agent, CI-976, that, in limited studies, has been shown to inhibit acyl CoA: cholesterol acyl transferase as well as LPCAT associated with the Golgi apparatus with an IC$_{50}$ of ~50 μM (39, 40). The results showed a dose dependent inhibition of the LPCAT activity of Prdx6; the percent inhibition was similar for Prdx6 assay at pH 4 and phosphorylated Prdx6 assay at pH 7 with 50% inhibition at approximately 10 μM (**Fig. 5**).

In order to evaluate LPCAT activity in a biologic tissue, we measured the incorporation of palmitoyl CoA into PC by isolated lung LBs. We have shown previously that these organelles contain relatively high levels of Prdx6 (19, 20). LBs from wild-type lungs incorporated labeled palmitate into PC (**Table 5**), an activity that requires the generation of LPC in the LBs and its subsequent acylation. Incorporation was similar in the presence or absence of added LPC, compatible with the presence of endogenous PLA$_2$ activity. There was markedly decreased incorporation of labeled palmitate into PC in the presence of MJ33, an inhibitor of the PLA$_2$ activity of Prdx6 and, therefore, an inhibitor of LPC generation. This decreased incorporation of palmitate into PC in the presence of MJ33 was reversed by the addition of exogenous LPC to the LB incubation medium (Table 5), compatible with the known effect of MJ33 as an inhibitor of PLA$_2$ activity. The presence of CI-976 also markedly inhibited the incorporation of palmitoyl CoA into PC, but, unlike the results with MJ33, there was no change in the inhibition with addition of LPC (Table 5). These results with CI-976 are compatible with the inhibition of LPCAT activity. There was essentially no incorporation of palmitate into PC by LBs isolated from Prdx6-null lungs.

The results with recombinant protein and with LBs show that Prdx6 expresses both PLA$_2$ and LPCAT activities and indicate that the PLA$_2$ activity can generate substrate (LPC) for subsequent acylation by LPCAT. One question is whether these two activities are linked or are essentially independent; i.e., is LPC generated by the PLA$_2$ reaction released from the protein or does it remain bound for subsequent acylation? To determine this, we measured the time course for incorporation of radiolabeled palmitate into unlabeled PC. Incorporation requires cleavage of the unlabeled fatty acid in the sn-2 position of PC and reacylation with labeled palmitoyl CoA. We have shown previously that Prdx6 does not express PLA$_1$ activity (19). We found a linear relationship between 10 and 90 min for incorporation of radiolabeled

TABLE 1. LPCAT activity of human and rat recombinant Prdx6

	LPCAT Activity (nmol/min/mg protein)			
	pH 4		pH 7	
	Human	Rat	Human	Rat
Prdx6	65 ± 0.6	68 ± 2	24 ± 4[a]	28 ± 2[a]
Phosphorylated Prdx6	652 ± 13	662 ± 14	662 ± 5	661 ± 7

Activity was determined by assay in the presence of LPC and ^{14}C-palmitoyl CoA. Values are mean ± SE for n = 3 independent experiments.

[a]$P < 0.05$ versus pH 4.
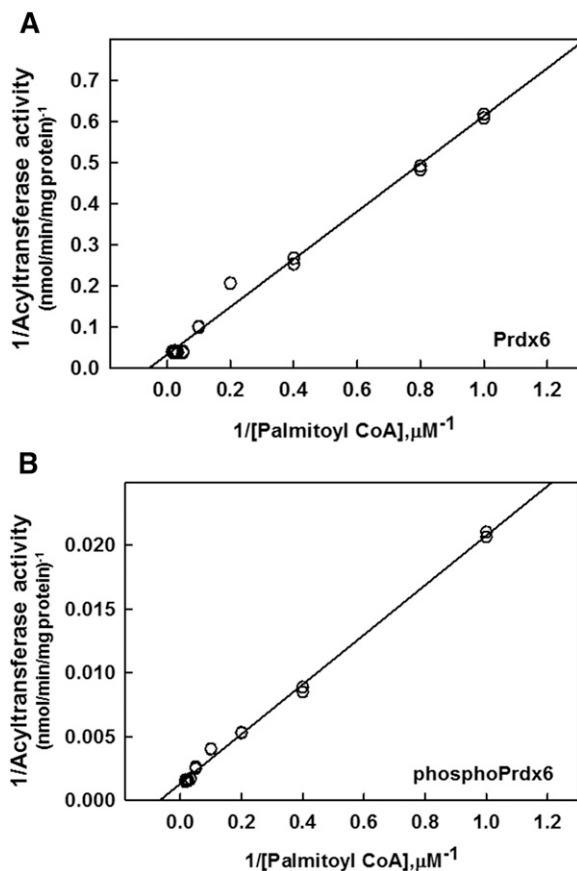
Fig. 4. LPC concentration dependence for Prdx6 LPCAT activity. Activity was determined as a function of palmitoyl CoA concentration that was varied from 1 to 100 μM. The results are plotted as double reciprocals. LPCAT activity was measured as in Fig. 3. A: Recombinant hPrdx6 assayed at pH 4. B: Recombinant phosphorylated hPrdx6 assayed at pH 7. The results are for two independent experiments as indicated by the plotted values; where only one symbol is evident, the two experiments gave identical values.

palmitate into PC for both Prdx6 (**Fig. 6A**) and phosphorylated Prdx6 (Fig. 6B), as well as for LBs (Fig. 6C). The addition of CI-976 (100 μM) inhibited incorporation of radiolabel into PC at 90 min of incubation by 87.1 ± 0.6% for Prdx6, 93.3 ± 0.2% for phosphorylated Prdx6, and 83.4 ± 1.3% for LBs (mean ± SE, n = 3), confirming that LPCAT activity is required for the measured reaction. If LPC were released from the enzyme prior to acylation, the expected plot would show a significant time lag pending build-up of the LPC concentration in the reaction cuvette. The linear

relationship for palmitate incorporation into PC using recombinant Prdx6 indicates that the LPC generated by PLA_2 activity remains bound to the protein for subsequent reacylation. A similar result was obtained for LB. Thus, the PLA_2 and LPCAT activities appear to represent a two-step coupled reaction.

We have previously described key amino acid motifs related to the PLA_2 and peroxidase activities of Prdx6 and have generated mutant proteins that express only one of these two activities. The C47S mutant protein does not express peroxidase activity (9, 10, 28, 41), but both PLA_2 and LPCAT activities are preserved (**Table 6**). For expression of only peroxidase activity, the mutants that were generated are H26A, S32A, and D140A, representing the catalytic triad for PLA_2 activity (2, 3). The D140A mutant protein retains full peroxidase activity, while the H26A and S32A mutants retain the ability to reduce short chain hydroperoxides, but cannot reduce phospholipid hydroperoxides, as they do not bind to the phospholipid substrate (2, 3, 9). These three mutant proteins lost PLA_2 activity, but retained LPCAT activity (Table 6). These results indicate that LPC, unlike DPPC, can bind to H26A and S32A Prdx6. Interestingly, mutation of Prdx6 to the H26A protein did not abolish its LPCAT activity (Table 6), even though this amino acid is a component of the LPCAT consensus sequence. We also generated D31A Prdx6, the other "required" amino acid component of the LPCAT consensus sequence. Although D31A Prdx6 has normal PLA_2 activity, it essentially lost its LPCAT activity (Table 6).

We evaluated these same mutations in endothelial cells that were infected with lentiviral vectors to produce mutant Prdx6 proteins. The cells utilized for the experiments were isolated from Prdx6-null mice so that the only Prdx6 protein present in the cells was generated as a result of lentiviral vector infection. Controls were Prdx6-null cells infected with either empty viral vector (no Prdx6) or wild-type Prdx6. Like the studies with recombinant proteins, C47S mutation of Prdx6 had no effect on PLA_2 or LPCAT activities (Table 6). Both H26A and D140A mutations in Prdx6 abolished PLA_2 activity, but LPCAT activity was unaffected. Similar to the effect on recombinant protein, D31A mutation of Prdx6 had no effect on PLA_2 activity, but abolished the LPCAT activity. The S32 mutation was not studied in endothelial cells.

Similar assays were carried out with homogenates of lungs isolated from Prdx6-null and Prdx6 C47S, H26A, and D140A mutant mice. The Prdx6-null lungs had very

TABLE 2. Kinetic constants for $aiPLA_2$ and LPCAT activities of recombinant hPrdx6

	K_m		V_{max}	
	PLA_2 (μM)	LPCAT (μM)	PLA_2 (nmol/min/mg protein)	LPCAT (nmol/min/mg protein)
Prdx6	350[a]	18	138[a]	30
Phosphorylated Prdx6	342[b]	15	1,815[b]	770

Prdx6 was assayed at pH 4; phosphorylated Prdx6 was assayed at pH 7. Values for LPCAT kinetic constants are derived from the mean of two independent experiments as shown in Fig. 4. Kinetic constants for PLA_2 activity have been reported previously.
[a]Data from (37).
[b]Data from (38).

TABLE 3. Head group specificity for LPCAT activity of recombinant hPrdx6

	LPCAT Activity (nmol/min/mg protein)	
	hPrdx6	Phosphorylated hPrdx6
Lysophosphtidylcholine	62.5 ± 2.3^a	651 ± 12^a
Lysophosphatidylethanolamine	3.0 ± 0.1	17 ± 3
Lysophosphatidylglycerol	2.4 ± 0.05	13 ± 2
Lysophosphatidylinositol	1.5 ± 0.04	10 ± 1
Lysophosphatidylserine	0.8 ± 0.08	6 ± 0.3^a

Activity was measured at pH 4 for Prdx6 and pH 7 for phosphorylated Prdx6 in the presence of ^{14}C-palmitoyl CoA and sn-1-palmitoyl lysophospholipid with the head group as indicated. Values are mean ± SE; n = 3 independent experiments.
[a]$P < 0.05$ versus all other values in the same column.

low levels of aiPLA$_2$ and LPCAT activities, as measured under these assay conditions (Table 6). The D140A and H26A mutant lungs retained LPCAT activity similar to wild-type, but had minimal aiPLA$_2$ activity. The C47 mutant lungs retained both activities. Thus, the lung studies gave results consistent with the results for recombinant proteins and lentivirus-infected cells. We have not yet generated mice with S32A or D31A Prdx6 mutations.

DISCUSSION

We have previously established that Prdx6 is a bifunctional enzyme with phospholipid hydroperoxide peroxidase and PLA$_2$ activities. These apparently disparate activities devolve from the binding site for phospholipids on Prdx6 that positions the substrate in relation to the two enzymatically active sites for the protein (3). The latter are the peroxidatic site centered on C47 at the bottom of a narrow cleft and S32 with adjacent H26 and D140 amino acids on the protein surface comprising the hydrolytic site for PLA$_2$ activity. Of course, Prdx6 can also reduce H$_2$O$_2$ and short chain hydroperoxides without the necessity for phospholipid "positioning." The present study demonstrates that Prdx6 also has acyl transferase activity that requires the presence of D31 in the protein. This latter site is part of a consensus sequence (^{26}HxxxxD^{31}) that is a hallmark for LPCAT enzymes (24). Amino acid D31 is also within what has been called a lipase motif (^{30}GxSxG^{34}), a consensus sequence expressed in many enzymes involved in lipid metabolism (35). The Prdx6 enzyme showed markedly
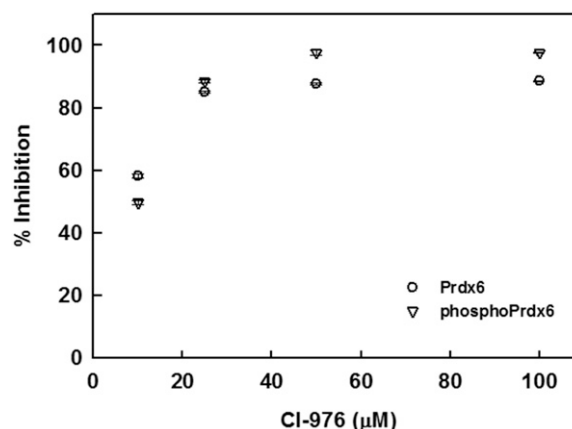

Fig. 5. Inhibition of Prdx6 LPCAT activity by CI-976. The concentration of inhibitor (CI-976) was varied from 10 to 100 μM in the presence of recombinant hPrdx6 or phosphorylated Prdx6. LPCAT activity was determined as in Fig. 3. Assay was at pH 4 for Prdx6 and pH 7 for phosphorylated Prdx6 (phophoPrdx6). The values, plotted as percent inhibition, are mean ± SE for n = 3 independent experiments.

greater affinity for acylation of LPC as compared with other lysophospholipid head groups. Thus, the Prdx6 LPCAT activity is predominantly lysophosphatidylcholine: palmitoyl CoA transferase.

This "new" activity of Prdx6 indicates that this protein can function in deacylation/reacylation reactions through sequential PLA$_2$ and LPCAT activities. Measurement of the incorporation of labeled palmitoyl CoA into PC in recombinant protein and in LBs showed a linear rate with no apparent lag, compatible with a coupled two-step reaction without the release of the intermediate LPC product. Thus, the enzyme, through its PLA$_2$ activity, can generate the substrate (LPC) for reacylation without releasing the product into the medium. This result is compatible with the demonstrated tighter binding for LPC, as compared with DPPC, to the Prdx6 protein (K_m 18 μM for LPCAT vs. 350 μM for PLA$_2$) (Table 2). For its PLA$_2$ activity, Prdx6 shows a relatively strong preference for PC, but does not show any preference for the fatty acyl group in the sn-2 position (19, 33). LPCAT shows a decided preference for LPC as the lysolipid and for palmitoyl CoA as the fatty acyl substrate. Therefore, this protein has the activity to remodel sn-2 unsaturated PC into sn-2 palmitoyl PC.

TABLE 4. Acyl specificity for LPCAT activity of recombinant hPrdx6

	LPCAT Activity (nmol/min/mg protein)	
	hPrdx6	Phosphorylated hPrdx6
Acetyl CoA	11 ± 1	14 ± 0.4
Palmitoyl CoA 16:0	64 ± 2^a	656 ± 7^a
Stearoyl CoA 18:0	6 ± 0.7	9 ± 0.3
Oleoyl CoA 18:1	13 ± 0.9	21 ± 2
Arachidonoyl CoA 20:4	8 ± 0.4	12 ± 0.9

Activity was measured with sn-1-^{14}C-palmitoyl LPC and the indicated acyl CoA at pH 4 for Prdx6 and pH 7 for phosphorylated Prdx6. Values are mean ± SE for n = 3 independent experiments.
[a]$P < 0.05$ versus all other values in the same column.

TABLE 5. LPCAT activity of isolated mouse lung LBs

	LPCAT Activity (nmol/hr/mg protein)	
	−LPC	+LPC
Wild-type	9.6 ± 0.2	10.1 ± 0.2
Wild-type + MJ33	0.09 ± 0.01^a	9.9 ± 0.8
Wild-type + CI-976	2.1 ± 0.1^a	2.0 ± 0.2^a
Prdx6 null	0.03 ± 0.01^a	0.05 ± 0.06^a

Activity was measured at pH 4 with 25–30 μg LB protein (incubation time 4 h) by incorporation of ^{14}C-palmitoyl CoA into DPPC in the presence or absence of LPC (150 μM). MJ33 was at 3 mol%; CI-976 was at 100 μM. Values are mean ± SE for n = 8 for wild-type −LPC, n = 6 for wild-type +LPC, and n = 3 for all other conditions.
[a]$P < 0.05$ versus corresponding wild-type.
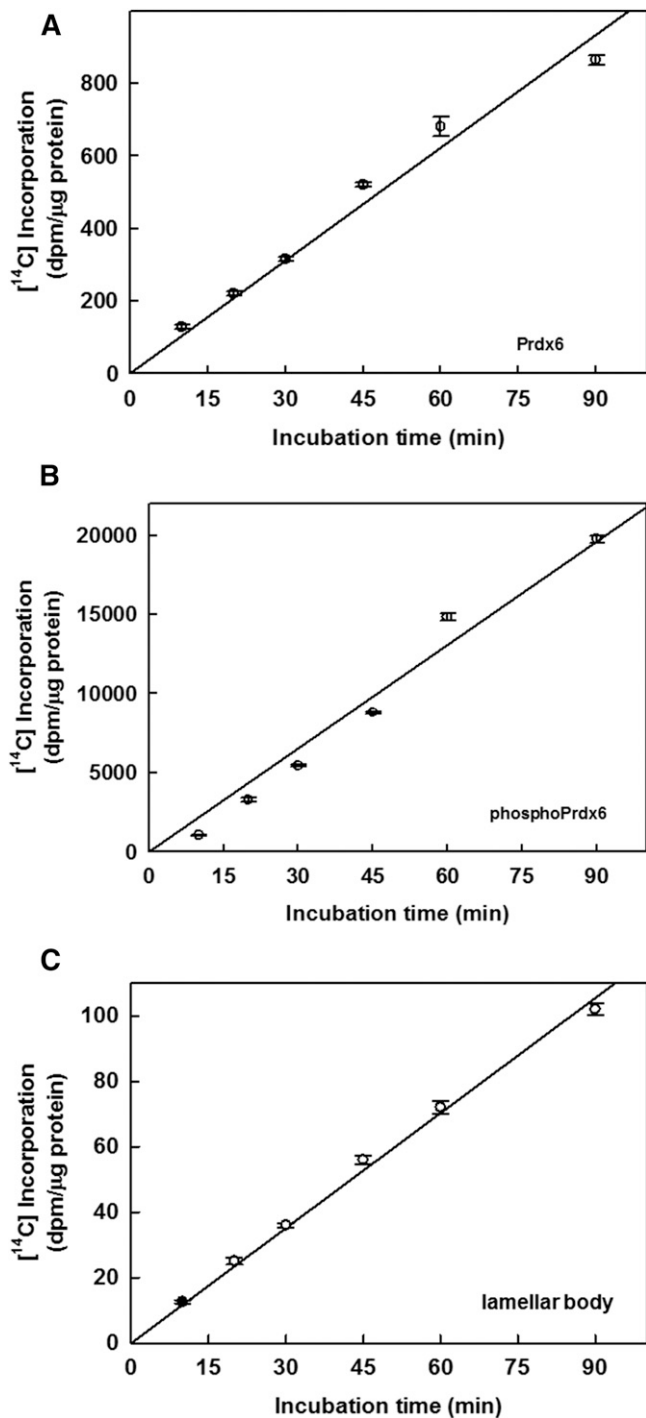
Fig. 6. Time dependence of deacylation/reacylation of PC by Prdx6. Incubation was for 10–90 min with ^{14}C-palmitoyl CoA (25 μM) and nonlabeled mixed unilamellar liposomes (DPPC, egg PC, phosphatidylglycerol, cholesterol; 0.5, 0.25, 0.1, and 0.15 mol fraction) in the absence of added LPC; total added lipid, 2 μM. The units are dpm incorporated into the PC fraction, normalized to Prdx6 or LB protein that was present in the assay. Incorporation of radiolabel requires deacylation of PC followed by reacylation with labeled palmitate. A: Recombinant hPrdx6 at 2.5 μg/ml, assay at pH 4. B: Recombinant human phosphorylated Prdx6 (phosphPrdx6) at 1 μg/ml, assay at pH 7. C: Mouse lung LB homogenate at 25 ug protein per milliliter, assay at pH 4. Values are mean ± SE for n = 3 independent experiments.

Previous studies have demonstrated that the lung LBs, a LRO, are a major site for phospholipid remodeling associated with the secretion/re-uptake of lung surfactant by the lung epithelium (12, 14, 15, 42). While a biosynthetic function for these organelles has not been investigated extensively, another LRO, the melanosome, is known to be the site for synthesis of melanin (43). The present study shows that the combined activity of PLA$_2$ and LPCAT in the lung LBs could account for the PC remodeling pathway that has been demonstrated in those organelles. The higher LPCAT activity of nonphosphorylated Prdx6 at pH 4 versus pH 7 is compatible with the acidic pH of lung LBs (44). The LBs also are the site for storage of newly synthesized lipids, as well as the site for accumulation of recycled lung surfactant (42). Our previous studies have demonstrated that the Prdx6 PLA$_2$ activity plays a major role in lung surfactant phospholipid remodeling, although the site for the required acyl transferase activity was not described (14). The present results confirm that the complete remodeling of PC into DPPC can occur within LBs.

Although Prdx6 is present in lysosomes and LROs such as LBs, the greater fraction of cellular Prdx6 is localized to the cytosol (11, 19, 41). Prdx6 does not bind to phospholipids at pH 7 (2, 3) and, thus, its cytosolic PLA$_2$ and LPCAT activities would be minimal in cells under normal conditions. On the other hand, Prdx6 binds more avidly to oxidized phospholipids at pH 7 (3) and, under conditions of oxidative stress, both PLA$_2$ and LPCAT activities would be expected to be expressed. The ability to bind to oxidized membrane phospholipids represents an important biological function for Prdx6, enabling it to serve as an important membrane "repair" enzyme (3, 9). Prdx6 can repair phospholipid hydroperoxides either by their reduction (peroxidase activity) or through deacylation/reacylation (PLA$_2$ plus LPCAT activities). Our previous studies using various models of oxidant stress have demonstrated that membrane repair in lung cells requires Prdx6 and that both the peroxidase and the PLA$_2$ activities are important (9, 10). While we have not yet studied the role of the LPCAT activity of Prdx6 in membrane repair, it would seem likely that this activity complements the PLA$_2$ activity in the repair process.

An additional mechanism, besides membrane phospholipid oxidation, for enabling the PLA$_2$/LPCAT activity of Prdx6 is phosphorylation of the protein through activity of the MAPKs, including Erk and p38 (22, 23). These kinases can be activated with inflammation resulting in the activation of Prdx6 under those conditions as a membrane repair enzyme. The proposed repair of oxidized membrane phospholipids via the reacylation reaction with Prdx6 LPCAT activity would result in a lipid with palmitate in the sn-2 position, while the original sn-2 fatty acid in the oxidized phospholipid presumably was unsaturated. The potential significance of this modification requires further study.

Four LPCAT enzymes, besides the present report, have been described during the past 10 years (24, 45), including two enzymes that are expressed at especially high levels in the lung (17, 46). These four proteins have significant differences as compared with the LPCAT activity of Prdx6.

TABLE 6. aiPLA$_2$ and LPCAT activities of wild-type and mutant recombinant Prdx6, lungs isolated from wild-type and mutant mice, and lentiviral vector-infected mouse pulmonary microvascular endothelial cells (MPMVECs)

	Recombinant Prdx6 (nmol/min/mg protein)		MPMVECs[a] (nmol/h/mg protein)		Lungs (nmol/h/mg protein)	
	aiPLA2	LPCAT	aiPLA2	LPCAT	aiPLA2	LPCAT
Wild-type	104 ± 3	65 ± 1	5.8 ± 0.3	3.4 ± 0.07	8.79 ± 0.73	5.39 ± 0.3
Prdx6$^{-/-}$	—	—	0.07 ± 0.14[b]	0.05 ± 0.07[b]	0.01 ± 0.11[b]	0.07 ± 0.02[b]
C47S	98 ± 1.4	59 ± 1.6	5.6 ± 0.2	3.2 ± 0.2	8.82 ± 0.28	5.10 ± 0.24
H26A	1 ± 0.02[b]	65 ± 0.6	0.01 ± 0.03[b]	3.1 ± 0.3	0.18 ± 0.03[b]	4.92 ± 1.1
S32A	0.9 ± 0.3[b]	66 ± 0.5	ND	ND	ND	ND
D140A	0.4 ± 0.1[b]	64 ± 2	0.09 ± 0.03	3.1 ± 0.9	0.03 ± 0.09[b]	5.05 ± 0.37
D31A	103 ± 2	0.4 ± 0.05[b]	5.45 ± 0.4	0.01 ± 0.01[b]	ND	ND

aiPLA$_2$ activity was measured with ^{3}H-DPPC; LPCAT activity was measured with 1-palmitoyl LPC plus ^{14}C-palmitoyl CoA. Values are mean ± SE for n = 3 independent experiments. ND, not determined.
[a] Prdx6-null MPMVECs were infected with lentiviral vector encoding wild-type or mutant Prdx6; Prdx6$^{-/-}$ represents infection with lentiviral vector alone.
[b] $P < 0.05$ versus corresponding wild-type.

All four of the previously described LPCAT enzymes are expressed predominantly in the ER, where they function in remodeling of newly synthesized lipids (24); an additional localization in lipid droplets has been shown for LPCAT1 and -2 (47). On the other hand, Prdx6 with its LPCAT activity is present in cell cytoplasm and LROs (19, 20). A second major difference between Prdx6 LPCAT and previously described LPCAT enzymes relates to substrate specificity. Unlike Prdx6 LPCAT, LPCAT2, -3, and -4 appear to prefer unsaturated fatty acyl substrates (24), and most phospholipids synthesized de novo do have an unsaturated fatty acid in the *sn*-2 position (42). LPCAT1 shows some specificity for saturated species of fatty acyl CoA, as described for Prdx6 LPCAT, but unlike Prdx6, can incorporate acetyl CoA into LPC. The substrate specificity of LPCAT1 appears to vary within different organs (48). Thus, this newly described activity of Prdx6 can be named LPCAT5.

The major site in the lung alveolar epithelial cell for surfactant PC synthesis is the ER, where a mixture of disaturated PC (predominantly DPPC) and PC containing an unsaturated fatty acid primarily in the *sn*-2 position are generated. LPCAT1 has been shown to participate in the remodeling of the unsaturated phospholipid to generate DPPC, and presumably this occurs in the ER where the enzyme is located (48). The present study demonstrates that the enzymes required for the remodeling of PC by deacylation/reacylation are also present in LBs. The precise relationship between LPCAT1 in the ER and Prdx6 LPCAT in LBs for providing lung surfactant DPPC remains to be determined.

Including this demonstration of LPCAT activity, Prdx6 has now been shown to express three distinct enzymatic activities involving phospholipids (PLA$_2$, LPCAT, and phospholipid hydroperoxide peroxidase) (28). These activities are linked mechanistically through the phospholipid binding site where the substrate is positioned in relation to the catalytically active sites of the protein (3). While no proteins have been described previously with both PLA$_2$ and LPCAT activities, a protein in peanuts and other seeds (oleosin) has been shown to express both acyl hydrolase (PLA$_2$) and monoacylglycerol acyl transferase activities (49). Oleosin contains acyl transferase and lipase motifs, as described for Prdx6, but appears to be involved predominantly in the pathway for synthesis of neutral lipids rather than phospholipids.

In summary, Prdx6 has lysophospholipid:acyl transferase activity that is relatively specific for LPC and palmitoyl CoA. This activity is coupled to the PLA$_2$ activity of Prdx6 without the release of intermediate products. Thus, Prdx6 can serve in the remodeling pathway of lipid synthesis, including the generation of DPPC for production of lung surfactant. Prdx6 represents the first LPCAT enzyme that has been shown with activity in cell cytoplasm and can function as a complete enzyme for the repair of peroxidized cell membrane phospholipids.

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