ABCA3 as a Lipid Transporter in Pulmonary Surfactant Biogenesis*‡

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ABCA3 protein is expressed predominantly at the limiting membrane of the lamellar bodies in alveolar type II cells, and mutations in the ABCA3 gene cause lethal respiratory distress in newborn infants. To investigate the function of ABCA3 protein, we generated Abca3-deficient mice by targeting Abca3. Full-term Abca3−/− newborn pups died within an hour after birth because of acute respiratory failure. Ultrastructural analysis revealed abnormally dense lamellar body-like organelles and no normal lamellar bodies in Abca3−/− alveolar type II cells. TLC and electrospray ionization mass spectrometry analyses of lipids in the pulmonary interstitium showed that phosphatidylcholine and phosphatidylglycerol, which contain palmitic acid and are abundant in normal surfactant lipids, were dramatically decreased in Abca3−/− lung. These findings indicate that ABCA3 plays an essential role in pulmonary surfactant lipid metabolism and lamellar body biogenesis, probably by transporting these lipids as substrates.

Pulmonary surfactant reduces surface tension at the air-liquid interface thereby preventing end-expiratory collapse of the alveoli of the lung. It is well known that surfactant deficiency causes respiratory distress syndrome (RDS) in preterm infants (1–5). Glucocorticoid is administered prenatally to prevent RDS by promoting maturation of the surfactant system, and surfactant replacement is widely used to treat RDS (6, 7). Pulmonary surfactant is composed mainly of lipids, mostly phospholipids, and specific proteins including surfactant proteins (SP)-A, SP-B, SP-C, and SP-D (8). Phosphatidylcholine (PC) and phosphatidylglycerol (PG), which contain palmitic acid, especially dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylglycerol, are uniquely abundant in the surfactant lipid component (9–11). The lipid and protein components are assembled in lamellar bodies, from which they are secreted into the alveolar space. However, the mechanism by which surfactant lipids are packaged in the lamellar body is not known.

We reported earlier that ABCA3, a member of the ATP-binding cassette (ABC) transporter superfamily, is expressed exclusively in alveolar type II cells and is localized mostly at the limiting membrane of the lamellar bodies in human lung (12). We also found that ABCA3 protein expression is dramatically increased just before birth and is up-regulated by glucocorticoids (13). As various members of the A subfamily of ABC transporters such as ABCA1 and ABCA4 have been shown to be involved in lipid transport (14–16), we proposed that ABCA3 functions as a transmembrane transporter of lipid components of pulmonary surfactant (12, 13). Recently, various mutations in the ABCA3 gene have been reported in full-term newborns with RDS (17) and in children with interstitial lung disease (18), indicating an important role of ABCA3 in human lung disease. However, the function of ABCA3 protein and the underlying mechanism of lung disease due to ABCA3 gene mutation remain unknown.

In the present study, to investigate the function of ABCA3 and its role in pulmonary surfactant biogenesis, we generated Abca3-deficient mice by homologous recombination and narrowed down the candidate substrates of ABCA3 protein by thin-layer chromatography (TLC) and electrospray ionization mass spectrometry (ESI/MS) analysis of lipids extracted from Abca3−/− lung. This approach should be useful in identifying the substrates of other orphan ABC transporters involved in lipid transport.

**EXPERIMENTAL PROCEDURES**

**Construction of the Targeting Vector and Generation of Abca3−/− Mice—**Mouse genomic DNA for the targeting construct for Abca3 was cloned by screening a mouse strain 129Sv λ genomic library (Stratagene) with a probe from the human ABCA3 cDNA fragment. Six clones of −19 kb containing the four exons encoding the first nucleotide-binding domain of
ABCA3 were isolated, restriction-mapped, and partially sequenced. An 889-bp PCR fragment and a 9.9-kb XhoI-Sall fragment were used as the 5′ short arm and 3′ long arm, respectively. These arms were inserted on either side of the PGK-neo cassette containing the neomycin phosphotransferase gene (Neo') under control of the mouse phosphoglycerate kinase (pGK) promoter in pBluescript. External to the 3′-region of homology with the target locus was placed a negatively selectable marker gene, driven diptheria toxin A (DT-A), under control of the MC1 promoter. 

E14 embryonic stem (ES) cells were cultured with the mouse embryonic fibroblasts as feeder cells in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum, 0.1 mM β-mercaptoethanol, and 1,000 units/ml leukemia inhibitory factor. For transfection, 1 × 10^7 ES cells were electroporated with 100 μg of the linearized targeting construct at 250 V, 500 microfarads in an 0.4-cm cuvette by using a Bio-Rad gene pulsar and then selected in the presence of 300 μg/ml G418 (Nacalai tesque). Colonies surviving positive/negative selection were isolated. Targeted ES clones were identified by PCR screening with primers ABCA3-S4 (5′-GCAATTTGTAATGACAGCTCATTC-3′) and Neo-S3 (5′-AGCTCATTCTCCCCACTGTG-3′) and verified by genomic Southern blot analysis using the 5′-external probe and the 3′-probe. Targeted ES clones were microinjected into C57BL/6J blastocysts, yielding chimeric mice that transmitted the targeted mutation through the germ line. Male chimeras were crossed with female C57BL/6J mice to generate heterozygous mice. Mice used in the experiments were obtained by intercrossing heterozygotes. The mice were maintained in the animal facility under specific pathogen-free conditions, and all animal experiments were approved by the Institutional Review Board of the Akita University School of Medicine.

DNA Blot Analysis—Genomic DNA from mouse tail was digested with BamHI for the 5′-probe and with Clai for the 3′-probe, separated by electrophoresis in 0.8% agarose, and transferred to Hybond-N+ membrane (Amersham Biosciences). Blots were hybridized with fluorescein isothiocyanate-labeled probe. The probe was detected by an alkaline phosphatase-conjugated anti-fluorescein isothiocyanate antibody (Amersham Biosciences).

PCR Analysis—Targeted ES clones were identified by PCR screening with primers ABCA3-S4 and Neo-S3 and AmpliTaq Gold DNA polymerase (PerkinElmer Life Sciences). The PCR condition was 94 °C for 9 min, 50 cycles at 94 °C for 15 s, 58 °C for 15 s, and 72 °C for 1 min, with a final elongation step at 72 °C for 7 min. Mouse genotypes were determined by PCR amplification of tail DNA. The wild-type Abca3 allele was characterized by amplification of a 477-bp fragment with sense primer ABCA3-S6 (5′-GCTCTTAGGAGACGAGTCTTGAAG-3′) and antisense primer ABCA3-AS9 (5′-TTGGAACACTCTCACGTAAG-3′). On the other hand, a 350-bp fragment of mutated ABCA3 was amplified with the same sense primer (ABCA3-S6) and antisense primer Neo-S4 (5′-ATTAAGGGCCAGCTCATTCT-3′) and ExTaq DNA polymerase (Takara). The PCR condition was 94 °C for 3 min, 35 cycles at 94 °C for 30 s, 62.5 °C for 30 s, and 72 °C for 30 s, with a final elongation step at 72 °C for 7 min.

Immunohistochemistry—The lungs were fixed overnight with 4% paraformaldehyde in 0.01 M phosphate buffer at 4 °C. The tissue was immersed in increasing concentrations of sucrose solution up to 20% and rapidly frozen in an embedding Tissue-Tek OCT compound (Sakura Finetechical) at −80 °C until used. 5-μm-thick sections were prepared with a cryostat (Leica) and transferred to aminopropyltriethoxysilane-coated Superfrost slide glass (Matsunami). The lung sections were blocked in 3% bovine serum albumin and 1% normal goat serum for 1 h at room temperature and incubated overnight at 4 °C in phosphate-buffered saline containing 3% bovine serum albumin, 1% normal goat serum, and 1:500-diluted anti-mouse F4/80 antigen rat antibody (MCA497R; Serotec). After incubation, the sections were immunostained by the Vectastain Elite ABC kit (Vector Laboratories) followed by incubation with 3,3′-diaminobenzidine (Sigma) for 5 min. The sections were mounted and observed with a light microscope (AxioCam HRc, Carl Zeiss).

Immunoblot Analysis—Crude lung proteins were prepared, and immunoblot analysis was performed as described previously (19). After blocking, the polyvinylidene difluoride membrane was incubated with 1:1000-diluted anti-mouse ABCA3 rabbit antibody (13), 1:1000-diluted anti-mouse SP-A rabbit antibody (sc-13977; Santa Cruz Biotechnology), 1:1000-diluted anti-mouse SP-B rabbit antibody (AB3780; Chemicon), 1:1000-diluted anti-mouse SP-C rabbit antibody (sc-13979; Santa Cruz Biotechnology), 1:1000-diluted anti-mouse SP-D rabbit antibody (AB3434; Chemicon), and 1:1000-diluted anti-β-tubulin isotype I + II mouse IgG₂b (T8535; Sigma) overnight at 4 °C. As the secondary antibody, 1:5000-diluted horseradish peroxidase-conjugated anti-rabbit Ig or 1:20000-diluted horseradish peroxidase-conjugated anti-mouse F(ab′)₂ (Amersham Biosciences) was used. Proteins were detected using an enhanced chemiluminescence (ECL) advance Western blotting detection kit (Amersham Biosciences).

Electron Microscopic Analysis—Lung tissue dissected from wild-type and Abca3−/− mice was fixed overnight at 6 °C in 0.1 M cacodylate buffer (pH 7.4) containing 3% glutaraldehyde. The fixed tissues were treated with 1% osmium tetroxide and 1.5% potassium ferrocyanide for 1 h and stained en bloc with 1% aqueous uranyl acetate for 2 h. The specimens were then dehydrated in ethanol and embedded in Spurr resin. 80-nm sections were cut on a Reichert Ultracut S ultramicrotome, collected on Formvar-coated copper oval slot grids, and stained with uranyl acetate and lead citrate. The sections were examined using an advanced transmission electron microscope H-7650 (Hitachi).

 TLC and Quantitative Analysis of Lipids—The isolated lung tissues were homogenized in distilled water by a Potter-Elvehjem homogenizer to obtain whole lung homogenate from wild-type and Abca3−/− mice. Bronchoalveolar lavage fluid (BALF) was prepared by bronchial intubation of phosphate-buffered saline in wild-type mice. As a practical alternative to BALF, the isolated lung tissue was cut into small pieces, digested by collagenase at 37 °C for 30 min, and centrifuged at 3000 rpm for 10 min, and the supernatant was collected as the interstitial fraction. Lipids were extracted by the method of Bligh and Dyer (20). For phospholipid analysis, aliquots of the solubilized lipids were developed on Silica gel 60 TLC plates
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**FIGURE 1. Generation of mice homozygous for null mutation in the Abca3 gene.** A, targeting strategy for Abca3. The targeting construct, normal Abca3 gene, and the resulting disrupted locus are depicted schematically. Positions of the neomycin phosphotransferase gene under transcriptional control of the phosphoglycerate kinase promoter (pGK-Neo) and the DT-A gene under the control of the MC1 promoter (pMC1-DT-A) are indicated. Successful targeting eliminates a 2948-bp fragment of the Abca3 gene containing the four exons encoding the first nucleotide-binding domain. The 5’- and 3’-probes for DNA blotting are indicated. Restriction enzymes: B, BamHI; C, ClaI; S, SfiI; X, XhoI. B, Southern blot analysis of genomic DNA from wild-type, heterozygous, and homozygous mice generated by a cross between two heterozygous Abca3 mice. (Abca3”). For confirmation of the correct recombination events at the 5’- and 3’-ends, genomic DNA isolated from tail tips was digested with BamHI and hybridized with 5’-probe or digested with ClaI and hybridized with 3’-probe, respectively. The genotypes are shown across the top, the restriction enzyme and the probe used are given below, and the sizes of hybridizing fragments are indicated to the side of the blots. C, genotyping analysis by PCR using the ABCA3-S6, ABCA3-AS9, and Neo-S4 primers. Ethidium bromide-stained PCR products from tail DNA shows the 477- and 356-bp fragments corresponding to the wild-type and the mutated alleles, respectively. DNA marker is 6X174 HaeIII-digested. D, immunoblot analysis of the lung tissue proteins from wild-type, Abca3”, and Abca3” mice probed with an anti-mouse ABCA3 antibody.

(Merc, 20 × 20 cm) using two-dimensional solvent systems; the first and the second chromatographic runs were performed with chloroform:methanol:7 M ammonia (65:30:4, v/v/v) and with chloroform:methanol:acetic acid:water (170:25:25:4, v/v/v) at −90° in the original direction. After development, the plates were sprayed with primuline (0.001% w/v in acetone-water (4:1)) and viewed under UV light (365 nm) for localization of separated compounds. Each lipid spot on the TLC plate was scraped off using a razor, and the spots of each phospholipid were then extracted from silica gel by chloroform/methanol/water (1:2:0.8, v/v/v). For measurement of the amount of inorganic phosphate in phospholipids, individual phospholipids extracted from silica gel were digested in perchloric acid (70%) for 90 min at 200°C. The inorganic phosphate was quantified colorimetrically (21). The calibration curves were prepared by using potassium dihydrogen phosphate as a standard. The amount of cholesterol contained in total lipids was measured using an enzymatic assay kit (Kyowa Medex) as described previously (22).

ESI/MS—The lipids of the interstitial fraction of lung tissues was extracted by the method of Bligh and Dyer (20) after the addition of 1,2-dimyristoleoyl-sn-glycero-3-phosphocholine (di-14:1 PC) (850346C; Avanti) and 1,2-dimyristoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (di-14:0 PG) (840445X; Avanti) as internal standards. The content of choline phospholipids in the interstitial fraction of lung tissues was analyzed by mass spectrometry. MS analysis was performed according to previous reports (23, 24) by using a Quattro Micro tandem quadrupole mass spectrometer (Waters) equipped with an ESI source. Phospholipid molecular species were separated and analyzed using normal-phase Cap-liquid chromatography ESI-MS with a silica column (Deverosil Si60, 150 × 0.3 mm inner diameter, 5 μm particle size, Nomura Chemicals, Nagoya, Japan). The samples were provided by UltiMate high performance liquid chromatography system (Dionex Corp.) into the electrospray interface at a flow rate of 4 μl/min. For both positive and negative ionization, the mobile phase consisted of acetonitrile/methanol (2:1) containing 0.1% ammonium formate (pH 6.4) (solvent A) and methanol/water (2:1) containing 0.1% ammonium formate (pH 6.4) (solvent B). At the beginning of the gradient, the mobile phase was 100% acetonitrile. After 5 min, the mobile phase was changed from 100% acetonitrile to solvent A-B (90:10). Solvent B was increased to 40% after 20 min. The mobile phase was then held constant for 15 min. The flow rate of the nitrogen drying gas was 12 liters/min at 80°C. The capillary and cone voltages were set at 3.7 kV and 30 V, respectively, argon at 3–4×10^4 torr was used as the collision gas, and a collision energy of 30–40 V was used to obtain fragment ions for precursor ions. The relationship between peak height and amount of PC and PG was examined using di-14:1 PC and di-14:0 PG.

Statistical Analysis—Two-tailed Student’s t test was used to determine the statistical significance of experimental results.

**RESULTS AND DISCUSSION**

**Generation of Abca3”−/− Mice**—We generated abca3-deficient mice by homologous recombination, which resulted in disruption of the four exons encoding the first nucleotide-binding domain of the ABCA3 protein. The targeting construct depicted in Fig. 1A was transfected into E14 ES cells, and a targeting frequency of 2% (4 of 196) was observed in clones that survived G418 and DT-A selection. Blastocyst injection of two clones resulted in the birth of seven high-percentage male chimeras. Transmission of the mutant allele was confirmed by PCR analysis and DNA blotting analysis using the 5’- and 3’-probes, which were set in the external region of the targeting
by the appearance of a white patch on the thorax signaling the inflated lung. On the other hand, all Abca3−/− mice initiated normal respiratory effort at birth but failed to inflate their lungs, remaining cyanotic and succumbing within an hour. At autopsy, the isolated Abca3−/− lung appeared to be congestive and poorly inflated, and wet lung weight was significantly increased by 32% compared with that of the wild type (Fig. 2, A and B).

No pathological conditions were observed in other organs of Abca3−/− pups. Histological examination of lungs from Abca3−/− mice revealed extensive atelectasis and pulmonary congestion (Fig. 2, C–F), findings consistent with acute RDS. In addition, immunoreactivity of F4/80 antigen, a selective marker of pulmonary macrophage, was not increased in the peripheral lung saccules of Abca3−/− mice compared with those of the wild type (supplemental figure). During the saccular period of fetal lung morphogenesis, the terminal buds dilate, the mesenchyme and epithelium become thinner, and there is an extensive ingrowth of pulmonary capillaries in close proximity to squamous respiratory epithelial type I cells as the gas exchange surface of the lung forms. At birth, the peripheral lung of wild-type mice consisted of saccules lined by both squamous type I

FIGURE 2. Morphogenesis of lung epithelium in Abca3-deficient mice. A, a pair of newborn littermates, wild-type (right) and Abca3−/− mice (left). The isolated lung tissue in Abca3−/− mice was congested compared with that in wild-type mice. B, body weight and wet lung tissue weight of wild-type and Abca3−/− mice. Each open circle represents a single pup. The numbers in parentheses at the bottom represent total numbers of pups and wet lungs weighed. The bracketed numbers at top represents average weight. The body weight of Abca3−/− mice (n = 22) was similar to that of wild-type mice (n = 26) at birth. However, the wet lung weight of Abca3−/− mice (n = 26) was significantly increased compared with that of wild-type mice (n = 30). N.S., not significant; *, p < 0.01, C–F, hematoxylin and eosin-stained histological sections of the lung tissue in wild-type (C and E) and Abca3−/− (D and F) littermates. Severe atelectasis, pulmonary congestion, reduced septonation, thickened mesenchyme, and columnar epithelium lining the peripheral saccules were observed in the Abca3−/− lung. Scale bars: 200 μm in C and D; 50 μm in E and F. G–I, consistent with the observed immaturity in the Abca3−/− lung, periodic acid-Schiff (PAS) staining demonstrated increased glycogen content in the bronchiolar epithelium of Abca3−/− mice (H and I) compared with control littermates (G). Scale bars: 50 μm in G and H; 25 μm in I. J, expression of the surfactant proteins in the Abca3−/− lung. Forty μg of total lung protein was subjected to Tris-Tricine SDS-PAGE and immunoblot analysis. Mature SP-C protein, but not pro-SP-C protein, was significantly decreased compared with wild type.

phenotype of Abca3−/− Mice—Heterozygous crosses showed that the distribution of the three genotypes (+/+ , +/− , −/−) was according to Mendelian inheritance (187:374:174). Heterozygous Abca3+/− mice were indistinguishable from normal littermates and showed normal growth, development, and reproduction. In contrast, no homozygous Abca3−/− mice survived the neonatal period but died of acute respiratory failure shortly after birth (Fig. 2A and supplemental video). The Abca3−/− pups were of normal size and body weight at birth compared with wild-type mice (Fig. 2B). Normal respiration was initiated at birth in both wild-type and Abca3−/− mice, which became pink and rapidly inflated their lungs as assessed

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construct (Fig. 1, A–C). To test for protein expression, we performed immunoblot analysis on proteins prepared from the lungs of wild-type, Abca3+/−, and Abca3−/− mice at birth, using the anti-mouse ABCA3 antibody. Immunoreactivity was detected in isolated lungs in wild-type and Abca3+/− mice but not in those of Abca3−/− mice (Fig. 1D). These results demonstrate that ABCA3 protein is absent in Abca3−/− lung due to knock-out of the Abca3 gene.

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cells and cuboidal type II cells, indicating structural maturation (Fig. 2, C and E). By contrast, in the lungs of Abca3−/− mice, the dilution of peripheral saccules was reduced, the mesenchyme remained thickened, and the peripheral saccules were lined by cuboidal epithelial cells with no typical squamous type I cells (Fig. 2, D and F), findings consistent with pulmonary immaturity (25). It is known that immature type II cells are glycogen-rich and that, as they differentiate, glycogen is converted into phospholipids and mobilized to lamellar bodies (26, 27). Sections from Abca3−/− lung display increased levels of periodic acid-Schiff staining in bronchiolar epithelium, indicated by the red deposit (Fig. 2, H and I), compared with their control littermates (Fig. 2G). These results suggest that maturation of epithelial cells is impaired in Abca3−/− lung.

It is well known that expression of surfactant proteins SP-A, SP-B, SP-C, and SP-D in alveolar type II cells in the peripheral lung normally increases prior to birth (28). In particular, the hydrophobic SP-B and SP-C play crucial roles in surfactant function and homeostasis (29); the proteins enhance the spreading, adsorption, and stability of surfactant lipids required

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for the reduction of surface tension in the alveolus, and mutations in the genes encoding SP-B and SP-C are associated with acute respiratory failure (30) and interstitial lung diseases (31), respectively. Therefore, we verified the expression levels of surfactant proteins using immunoblot analysis. Expressions of SP-A, prepro-SP-B, pro-SP-C, and SP-D proteins were readily detected in lung homogenate of Abca3−/− mice and were indistinguishable from those in wild-type mice. In contrast, mature SP-C protein was not detected in lung homogenate of Abca3−/− mice (Fig. 2f), suggesting that disruption of the Abca3 gene causes aberrant processing of pro-SP-C resulting in a deficiency of mature SP-C protein in Abca3−/− lung. In this regard, Abca3−/− mice resemble SP-B−/− mice and human infants with hereditary SP-B deficiency, in both of which mature SP-C is deficient (32, 33).

Ultrastructural Analysis of Alveolar Type II Cells in Abca3−/− Mice—At the ultrastructural level, alveolar type II cells with apical microvilli were found to contain numerous lamellar bodies in control wild-type mice (Fig. 3A). In contrast, fully formed lamellar bodies were not detected, and only lamellar body-like organelles were observed in alveolar type II cells of Abca3−/− mice. Lamellar body-like organelles are smaller than those observed fully formed in wild-type mice and have eccentrically placed, dense inclusions (Fig. 3, B and C). This is consistent with observations in the lung tissue of a newborn patient with fatal RDS with ABCA3 mutation (17) and in differentiating human fetal lung alveolar type II cells in which endogenous ABCA3 is knocked down with small interfering RNA (34). Clearly, ABCA3 protein is involved in the metabolism of the lipid components of pulmonary surfactant and is critical in surfactant biogenesis and lamellar body formation.

Lipid Composition in Interstitial Fraction of Abca3−/− Lung—To clarify the function of ABCA3, we compared the composition of surfactant lipids in lung tissues of wild-type and Abca3−/− mice. To determine the composition of secreted surfactant lipids, we attempted to obtain the lipids in BALF from both wild-type and Abca3−/− mice at birth. Although BALF could be obtained from wild-type and Abca3−/− mice, we were unable to obtain BALF from Abca3−/− mice, because injection of phosphate-buffered saline into alveolar space was impossible, perhaps because of increased surface tension at the alveolar air-liquid interface or morphological abnormalities in the peripheral lung. Therefore, rather than BALF, we examined the interstitial fraction of lung tissue prepared by collagenase digestion. Crude lipids extracted from the interstitial fraction were separated by two-dimensional TLC analysis (Fig. 4A). By quantification of phospholipid phosphorus, the amounts of PC and PG in the interstitial fraction of Abca3−/− lung were dramatically decreased 63 and 96%, respectively, compared with those of wild-type mice. In addition, the ratio of these lipids relative to total phospholipids in Abca3−/− lung also was significantly lower than that in wild-type mice (PC, 55.7% in Abca3−/− versus 69.2% in wild type, p < 0.001; PG, 0.6% in Abca3−/− versus 4.5% in wild type, p < 0.001). The amount of phosphatidylethanolamine (PE) was moderately but significantly decreased 45% in Abca3−/− mice compared with that of wild type, but the ratio of PE relative to total phospholipids was not significantly different in Abca3−/− and wild-type lung (11.1 versus 9.4%, respectively; p = 0.478). Thus, it was unclear whether the decrease in the amount of PE in Abca3−/− lung was significant. On the other hand, the amounts of total cholesterol and other phospholipids from Abca3−/− lung were not different from those of wild-type lung (Fig. 4A). These results demonstrate that secretion of PC and PG is impaired in Abca3−/− lung, suggesting these phospholipids as substrates of the ABCA3 transporter. Surfactant contains a large amount of PC (70–80% of total phospholipids); PG, which accounts for less than 2% of total phospholipids in other tissues, is the second major phospholipid in surfactant, accounting for as much as 10% of phospholipid content (9, 11). Especially PC and PG, both of which contain palmitic acid, are abundant in lamellar bodies, accounting for 70% of total PC and 50% of total PG, respectively (10, 11), and are crucial in reducing surface tension at the alveolar air-liquid interface. Very recently, the ratio of PC in total surfactant phospholipids extracted from BALF in patients with ABCA3 gene mutation has been reported to be decreased compared with control (35), which is consistent with our data.

To further determine which species of PC and PG are decreased, ESI/MS analysis was conducted on the interstitial fraction from wild-type and Abca3−/− lung tissue. Among the PC species from Abca3−/− lung, 14:0−16:0 PC, 16:0−16:1 PC, and 16:0−16:0 PC were dramatically decreased by about 80%, whereas 16:0−18:1 PC and 16:0−22:6 PC were moderately and significantly decreased by 30 and 60%, respectively, compared with wild-type mice (Fig. 4B). Among the PG species in Abca3−/− lung, 16:0−16:1 PG, 16:0−16:0 PG, 16:0−18:1 PG, 16:0−20:4 PG, and 16:0−22:6 PG were dramatically decreased by more than 80% compared with wild-type lung (Fig. 4C). On the other hand, differences in the amounts of PE species were not obvious between Abca3−/− and wild-type lung (data not shown). These results demonstrate that PC and PG, both of which contain palmitic acid, are specifically decreased in Abca3−/− lung.

Very recently, Fitzgerald et al. (36) reported ABCA3 knockout mice showing a phenotype similar to that of our Abca3−/− mice. They analyzed the lipid components of total lung
homogenates at embryonic day 18.5, when expression of endogenous ABCA3 protein is extremely low (13). We analyzed the interstitial fraction, which represents secreted surfactant, just after birth. In addition, by analyzing the acyl chain using tandem mass spectrometry analysis, we clearly show that PC and PG, especially those species containing palmitic acid, are dramatically decreased. We also found that mature SP-C protein was not detected in lung homogenate of Abca3−/− mice, suggesting that disruption of the Abca3 gene causes aberrant processing of pro-SP-C. In addition, we examined the effect of overexpression of ABCA3 protein on phospholipids levels in human lung adenocarcinoma A549 cells and found that the choline-phospholipid level in intracellular LAMP3-positive vesicles in A549 cells stably expressing ABCA3 protein is increased compared with that in untransfected A549 cells,3 which supports these data. However, the effect of overexpression of ABCA3 on the PG level remains uncertain, as PG barely exists in A549 cells.

In summary, we have determined by TLC and ESI/MS analysis of lipids extracted from Abca3−/− lung that PC and PG, which contain palmitic acid, are candidate substrates of ABCA3 protein. This approach should be helpful in the investigation of substrates of other orphan ABC transporters involved in lipid transport.

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FIGURE 4. Phospholipid composition of lung interstitial fractions in wild-type and Abca3−/− mice. A, representative TLC analysis of phospholipids extracted from the lung interstitial fraction in wild-type and Abca3−/− mice. The amount of inorganic phosphate in phospholipids was quantified colorimetrically. Data are represented as means ± S.D. (n = 3). *, p < 0.05; **, p < 0.01 versus wild-type. N.S., not significant. Abbreviations for phospholipids shown are as follows: PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid; CL, cardiolipin; SM, sphingomyelin. B and C, representative ESI/MS analysis of phospholipids extracted from the lung interstitial fraction in wild-type and Abca3−/− mice. Positive-ion ESI/MS spectra of phosphatidylcholine molecular species (B) and negative-ion ESI/MS spectra of phosphatidylglycerol molecular species (C) in lipid extracts of the lung interstitial fraction in wild-type mice and Abca3−/− mice are shown. Aliquots of chloroform/methanol (2:1 (v/v)) extracts were provided by the UltiMate HPLC system into the electrospray interface. Individual molecular species were identified using tandem mass spectrometry. The internal standards (I.S.) were 14:1–14:1 PC (m/z 674.5) and 14:0–14:0 PG (m/z 665.4) (Avanti). Data are represented as means ± S.D. (n = 5). *, p < 0.05; **, p < 0.01 versus wild type.
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