ABCA3 is a lamellar body membrane protein in human lung alveolar type II cells[1]

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Abstract The ABCA3 gene, of the ABCA subclass of ATP-binding cassette (ABC) transporters, is expressed exclusively in lung. We report here the cloning, molecular characterization, and distribution of human ABCA3 in the lung. Immunoblot analysis using the specific antibody reveals a 150-kDa protein in the crude membrane fraction of human lung. Immunohistochemical analyses of alveoli show that ABCA3 is expressed only in the type II cells expressing surfactant protein A. At the ultrastructural level, ABCA3 immunoreactivity was detected mostly at the limiting membrane of the lamellar bodies. Since members of the ABCA transporter family are known to be involved in transmembrane transport of endogenous lipids, our findings suggest that ABCA3 plays an important role in the formation of pulmonary surfactant in type II cells.

Key words: ATP-binding cassette transporter; Alveolar type II cell; Lipid transport; Surfactant; Lamellar body; Surfactant protein A

1. Introduction

The ATP-binding cassette (ABC) transporter superfamily is one of the largest gene families, encoding highly conserved proteins involved in energy-dependent transport of a variety of substrates across membrane, including ions, amino acids, peptides, carbohydrates, and lipids [1]. Among the several subclasses of ABC transporters, the ABCA subclass [2] has received considerable attention because mutation of the ABCA1/ABC1 or the ABCA4/ABCR gene causes Tangier disease accompanying high density lipoprotein deficiency [3,4] or Stargardt's macular dystrophy [5] in human, respectively. In addition to ABCA1 and ABCA4, five other members, ABCA2/ABC2 [6], ABCA3/ABC3 (ABC-C) [7,8], ABCA6 [9], ABCA7 [10], and ABCA8 [9], have been shown to belong to the ABCA subclass to date. ABCA1 and ABCA4 are proposed to be transmembrane transporters for intracellular cholesterol/phospholipids [3,4,11] and protonated *N*-retinylidene-phosphatidylethanolamine [12], respectively. ABCA2, ABCA3, and ABCA7 mRNA levels have been reported to be upregulated by the sustained cholesterol influx mediated by modified low density lipoprotein [13,14], suggesting that ABCA transporters including ABCA3 are involved in transmembrane transport of endogenous lipids [4].

ABCA3, which was originally cloned from a cDNA library constructed from human medullary thyroid carcinoma cell line [7] or from human genomic DNA by exon-trapping [8], is expressed predominantly in the human lung [7,8]. However, the distribution and function of ABCA3 in the lung remain unknown, and it has not been cloned from lung tissues.

In the present study, we cloned ABCA3 from human lung and generated a specific antibody against it, and found that the 150-kDa ABCA3 protein is expressed predominantly at the limiting membrane of the lamellar bodies in the type II cells in human lung alveolar structures. The data suggest that ABCA3 may play an important role in the formation of pulmonary surfactant, which is composed mainly of phospholipids and specific surfactant proteins [15].

2. Materials and methods

2.1. Construction of human ABCA3 expression vector

7.2×10^5 plaques of a human lung cDNA library (Stratagene) were screened under the standard hybridization conditions [16] using DNA fragments of mouse EST clones AW900257 and AA638829, which contain the regions corresponding to the 5′-untranslated region to nucleotide +1020 (relative to the putative translation start site) and nucleotide +4820 to the 3′-untranslated region of human ABCA3 cDNA, respectively, as probes. Three of the positive 71 clones, λhABCA3-5-1, λhABCA3-5-4, and λhABCA3-3-17, were subcloned and sequenced. In addition, to obtain the DNA fragment covering the middle region of human ABCA3, the DNA fragment from +2475 to +4313 was amplified by polymerase chain reaction (PCR) using human lung cDNA (Clontech), subcloned, and sequenced. Using these DNA fragments, the expression vector (pCMVhABCA3) carrying the full-length cDNA of human ABCA3 was constructed.

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[1] The nucleotide sequence for human ABCA3 has been deposited in the DDBJ/EMBL/GenBank nucleotide sequence database under accession number AB070929.

Abbreviations: ABC, ATP-binding cassette; PCR, polymerase chain reaction; SP-A, surfactant protein A; PBS, phosphate-buffered saline; DAB, 3,3′-diaminobenzidine-4HCl

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2.2. Tissue preparation, cell culture, and transfection

Human lung tissue was obtained from adult patients undergoing surgical removal for various reasons. Culture and transfection of HEK 293 cells were carried out as previously described [17]. 2 μg of pCMVhABCA3 or pCMV vector was transfected into cells with Lipofectamine and Opti-MEM I (Life Technologies, Inc.), according to the manufacturer's instructions.

2.3. Crude membrane preparation

Crude membrane of transfected HEK 293 cells or human lung tissue was prepared as previously described [6,17]. Briefly, for transfected HEK 293 cells, 3 days after transfection with pCMVhABCA3 or pCMV vector alone, the cells were washed, suspended in buffer A consisting of 50 mM Tris (pH 7.5) and 1 mM EDTA containing protease inhibitor cocktail (10 μl/ml) (Sigma), homogenized, and then ultracentrifuged at 100 000 × *g* at 4°C for 1 h. The pellets were resuspended in 500 μl of buffer A and stored at −80°C until immunoblot analysis. For lung tissue, the tissue in buffer A (10% volume) containing protease inhibitor cocktail (10 μl/ml) was homogenized in a Teflon pestle-glass homogenizer on ice and centrifuged at 800 × *g* for 7 min at 4°C. The supernatant was then subjected to ultracentrifugation, and the pellet was resuspended and stored as described above. Protein concentrations were determined using the BCA assay (Pierce, Rockford, IL, USA).

2.4. Primary antibodies

The specific antibody for ABCA3 was raised in a rabbit against the synthetic peptide corresponding to 13 C-terminal amino acid residues (FAHLQPPTAEEGR) of human ABCA3, which differs construc-

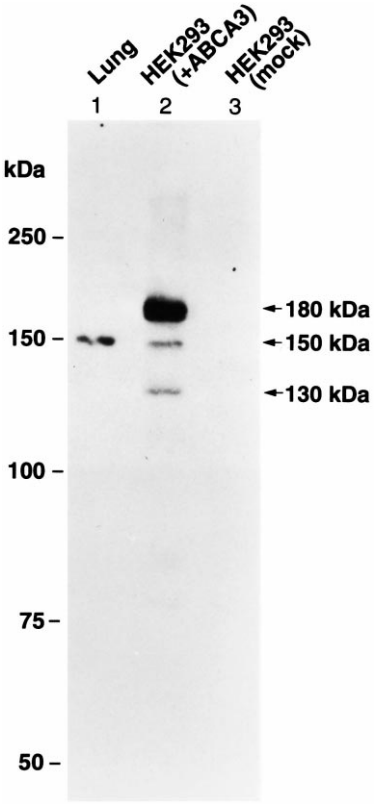

Fig. 1. Immunoblot analysis of ABC3 protein. Membrane proteins prepared from human lung tissue (20 μg) (lane 1) and HEK 293 cells transfected with pCMVhABCA3 (10 μg) (lane 2) and empty vector (pCMV; mock) (10 μg) (lane 3) were electrophoresed on a 7% SDS–polyacrylamide gel. For immunoblot analysis, an anti-ABCA3 rabbit antibody and a horseradish peroxidase-conjugated anti-rabbit IgG were used as the primary and secondary antibodies, respectively. Proteins were detected using an enhanced chemiluminescence system. Molecular weights of markers are indicated on the left, and the detected ~180-kDa, 150-kDa, and 130-kDa bands are indicated by arrows.

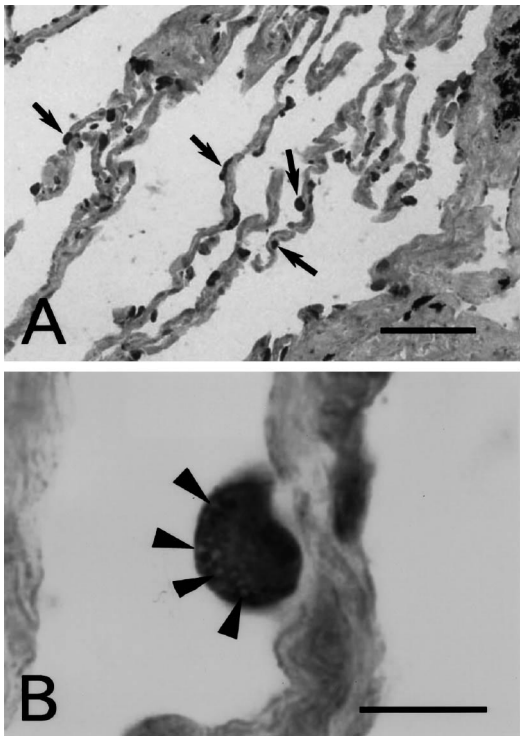

Fig. 2. Immunohistochemical labeling of ABCA3 by DAB reaction of human lung tissue cryosections. A: Immunoreactive round or cuboid cells (arrow) are scattered throughout the alveoli. B: A representative immunoreactive cell; vesicle-like structures are strongly stained on the surface (arrowhead). Scale bar, 10 μm.

tively from other ABCA transporters and any other protein from the sequence databases. The antibody was purified using affinity chromatography (HiTrap Protein G, Amersham Pharmacia Biotech). For double-immunofluorescence staining, a mouse monoclonal IgG antibody against human surfactant protein A (SP-A, PE-10, Dako, Japan) or a mouse monoclonal antibody against Hsp-60 of mitochondria (MBL, Nagoya, Japan) was used.

2.5. Immunoblot analysis

The crude membrane proteins prepared from transfected HEK 293 cells (10 μg) and human lung tissue (20 μg) were boiled in SDS reducing sample buffer, electrophoresed on 7% SDS–polyacrylamide gel, and transferred to a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech) as previously described [6]. After blocking and washing [6], the membrane was incubated with 1:1000 diluted anti-ABCA3 antibody for 2 h at room temperature. After washing, the membrane was incubated with 1:5000 diluted horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Pharmacia Biotech) for 1 h, followed by washing [6]. Proteins were detected using an enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech).

2.6. Immunohistochemistry

Portions of human lung tissue were fixed by immersion with 4% paraformaldehyde containing 0.1% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.2) for 1 day at 4°C. After washing with 0.1 M PB, the fixed tissues were trimmed to smaller pieces, incubated for two nights in 30% sucrose in 0.1 M phosphate-buffered saline (PBS), and cut into 8 μm thick sections on a cryostat (MICROM HM 500; MICROM, Heidelberg, Germany). The sections were rinsed with PBS, preincubated with 10% normal goat serum in PBS, and then incubated with 1:10 000 diluted anti-ABCA3 antibody. After incubation, the sections were immunostained by the avidin–biotin complex methods (Vector, Burlingame, CA, USA), and were subsequently incubated with a 3,3′-diaminobenzidine-4HCl (DAB) kit (Vector) for about 5 min. The sections were mounted and observed with an Olympus AX-70 microscope (Olympus, Tokyo, Japan).

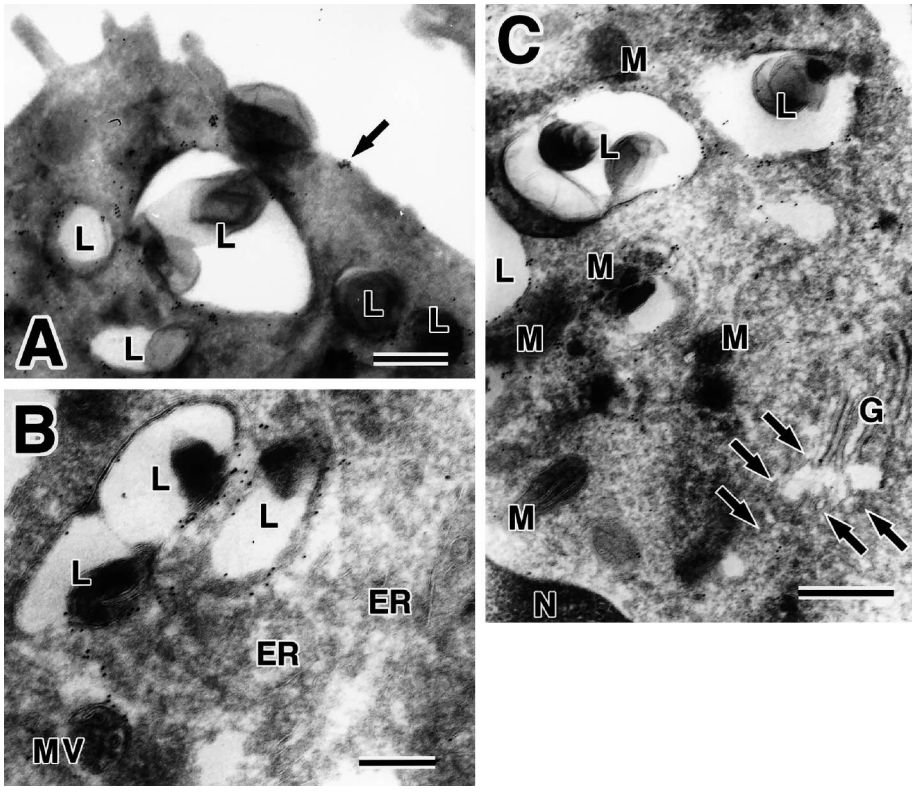

Fig. 3. Immunogold labeling of ABCA3 in cryo-ultrathin sections of human alveolar type II cells. A: Many gold labelings appear on the limiting membrane of lamellar bodies. A few gold particles are localized in a patch on the plasma membrane (arrow). Scale bar, 500 nm. B: Multivesicular bodies near the lamellar bodies also are labeled on the limiting membrane. Endoplasmic reticulum is not labeled. Scale bar, 250 nm. C: Golgi cisternal edge (indicated by arrows) is labeled only sparsely. Mitochondria and nucleus are not significantly labeled. Scale bar, 250 nm. L: lamellar body, MV: multivesicular body, ER: endoplasmic reticulum, G: Golgi apparatus, M: mitochondria, N: nucleus.

2.7. Immunoelectron microscopy

Portions of human lung tissue were incubated for more than 3 days in cold 1.84 M sucrose in 0.1 M PB containing 20% polyvinyl pyrrolidone. After being cut into very small pieces, they were rapidly frozen in liquid nitrogen at −196°C. Frozen ultrathin sections (100 nm) were cut with a Leica Ultracut S/FCS (Vienna, Austria). The sections were picked up on a formvar-carbon-coated nickel grid, incubated with 2% gelatin in PBS containing 10 mM glycine, and allowed to react overnight with 1:1000 diluted anti-ABCA3 antibody. The tissues were washed five times with glycine-PBS containing 0.5% gelatin and then for 2 h with 10 nm colloidal gold-labeled goat anti-rabbit IgG antibody (2 μg/ml) (British Biocell International, UK). After being washed again, the sections were post-fixed in 0.1% glutaraldehyde, stained with 2% uranyl acetate, embedded in polyvinyl alcohol, and observed with a Jeol JEM-1200 EX II electron microscope (Jeol, Tokyo, Japan).

2.8. Confocal laser scanning microscopy

Tissues with brief fixation in 4% buffered paraformaldehyde were embedded in OCT compound, snap frozen in acetone–dry ice, and stored at −80°C until sectioning. A double indirect immunofluorescence method was applied to sections of 4 μm or 30 μm thickness. The sections were incubated overnight at 4°C with a combination of 1:500 diluted anti-ABCA3 antibody and 1:100 diluted anti-SP-A antibody or 1:200 diluted anti-Hsp-60 antibody as primary antibodies. After washing several times, the sections were incubated with a mixture of two secondary antibodies consisting of 1:100 diluted each of fluorescein isothiocyanate-labeled goat anti-rabbit IgG and Texas red-labeled horse anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA), for 40 min at room temperature in the dark. Nuclear counterstaining was done for 15 min at room temperature with 0.01% TOTO-3 iodide (Molecular Probes, Eugene, OR, USA). The sections were examined with a confocal laser scanning microscope (TC-SP; Leica, Heidelberg, Germany) equipped with argon and argon–krypton laser sources.

3. Results and discussion

Screening a human lung cDNA library and amplifying human lung cDNA using PCR, we cloned a full-length human ABCA3 cDNA encoding a 1704 amino acid protein. The amino acid sequence predicted from the composite sequence (from nucleotide −42 to +5196 relative to the putative translation start site) is identical to that cloned from the human medullary thyroid carcinoma cell line [7], while the 196th amino acid residue (proline) is different from that (leucine) of the ABCA3 protein cloned from genomic DNA by exon-trapping [8]. There are also two polymorphic sites without amino acid substitution at nucleotide +798 (T) and +1176 (T) that are different from the two sequences previously reported (C at both sites) [7,8].

ABCA3 protein then was analyzed by immunoblotting using the antibody generated against human ABCA3. A single band at 150 kDa was detected from the crude membrane fraction of human lung tissue (Fig. 1, lane 1). To ascertain specificity of the antibody, crude membrane fractions of transfected HEK 293 cells were examined. Four bands, two close bands at about 180 kDa and one at 150 kDa and 130 kDa, were detected in HEK 293 cells transfected with ABCA3 expression vector (pCMVhABCA3) (Fig. 1, lane 2), but no band was detected in cells transfected with vector alone (pCMV) (Fig. 1, lane 3). These results indicate that ABCA3 is a 150-kDa protein in the lung. The reason why several bands are detected in HEK 293 cells and the molecular size detected in the lung is smaller than that predicted from the deduced ami-

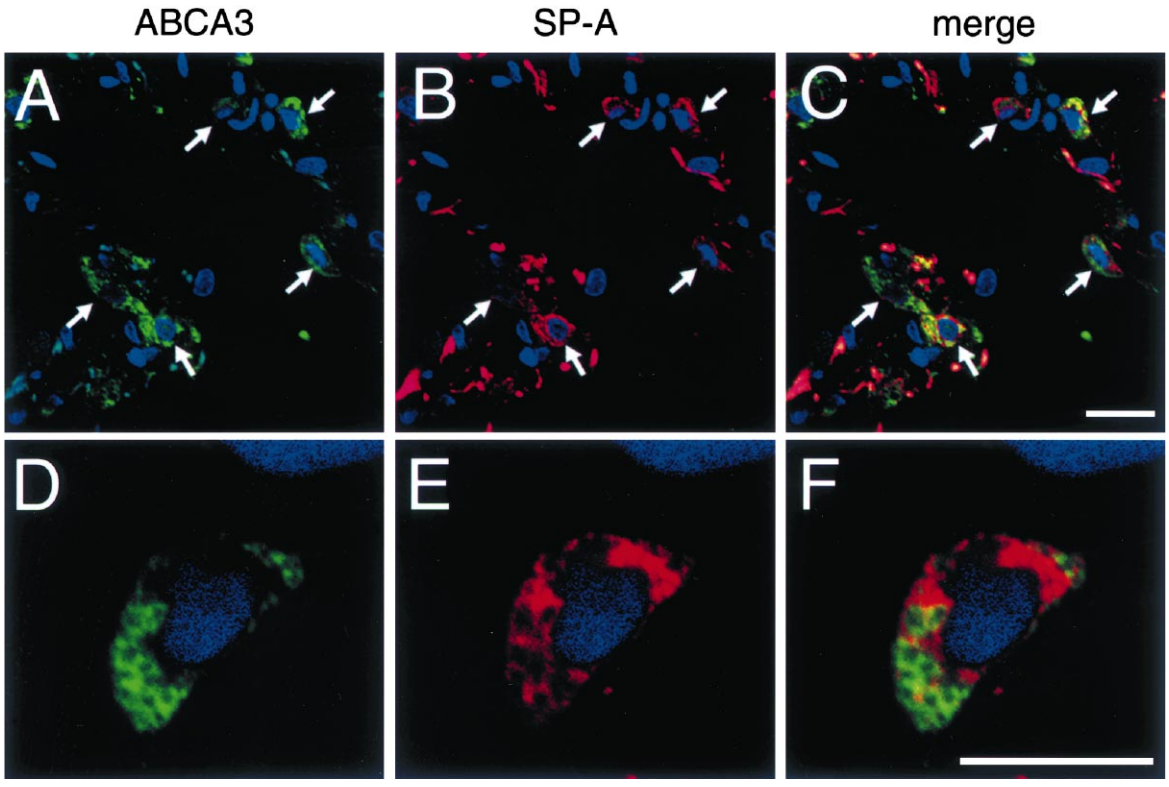

Fig. 4. Immunohistochemical labeling of ABCA3 and SP-A of human lung tissue cryosections. A–C: Low power view of alveolar walls of human lung. Type II cells, in which both ABCA3 (A) and SP-A (B) are present, are located on the alveolar wall, as indicated by arrows. Merged picture is shown in C. The many cells not labeled with ABCA3 or SP-A (blue) are nuclei. The structures of yellow color in C which correspond to elastic fibers of alveolar walls or perivascular elastic fibers are also observed. Scale bar, 20 μm. D–F: High power view of a representative type II cell. The surrounding structure in green fluorescence (ABC3) (D) does not completely overlap the red fluorescence mass (SP-A) (E) in the merged picture (F). Scale bar, 10 μm. Green fluorescence shows localization of ABC3 protein, red fluorescence SP-A, and blue fluorescence nuclei.

no acid sequence (191 kDa) is unknown, but may be post-translational modification.

We then examined the expression of ABCA3 in human lung by DAB reaction. ABCA3 immunoreactivity appears to be very intense in the comparatively large, round or cuboid cells scattered in the alveolar epithelium (Fig. 2A). Where vesicle-like structures appear in the cells, their surfaces are stained especially strongly (Fig. 2B).

To further examine the intracellular localization of ABCA3, an immunoelectron microscopic study with colloidal gold label on ultrathin cryosections of human lung was performed. Immunogold particles were observed mainly in cells with apical microvilli on the plasma membrane (Fig. 3A), a feature of type II cells. Major labelings are associated with lamellar bodies specific to type II cells, and most are localized exclusively on the limiting membranes and not inside the lamellar bodies (Fig. 3A–C). Immunolabelings also appear on the surfaces of multivesicular bodies (Fig. 3B), and there are sparse patches of gold particles on the cell membrane (Fig. 3A). Golgi cisternal edges and Golgi vesicles (Fig. 3C) also are labeled sparsely. The endoplasmic reticulum, mitochondria, and nucleus all are devoid of gold particles (Fig. 3B,C).

To confirm that ABCA3 is expressed in type II cells, a double-immunofluorescence staining experiment was performed using both the anti-ABCA3 antibody and an antibody against SP-A, which is known to be expressed in both alveolar type II cells and bronchiolar Clara cells in the lung [15]. As shown in Fig. 4, cells double-immunolabeled for ABCA3 and SP-A are scattered throughout the alveolus, clearly showing that ABCA3 is expressed in alveolar type II cells. Closer examination reveals immunoreactivity for ABCA3 and SP-A mainly in the cytosol, but with different distribution patterns. ABCA3 consistently stained a ring-like structure (Fig. 4B), probably reflecting the rim of the lamellar body, which often lacked reactivity to SP-A. This is consistent with a previous report that SP-A immunoreactivity is present abundantly on rough endoplasmic reticulum and the Golgi apparatus and associated small vesicles but at low levels on lamellar and multivesicular bodies in type II cells [18]. We further confirmed that Hsp-60 is localized only in mitochondria, which are clearly distinct from the ABCA3-labeled ring-like structures in type II cells (data not shown). The expression of ABCA3 in Clara cells in the airway was not examined in this study.

Pulmonary surfactant, composed mainly of phospholipids and specific surfactant proteins, reduces the surface tension at the alveolar air/liquid interface, and thereby prevents the lungs from collapsing [15]. The main phospholipid component, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, is unique in that two acyl chains in the molecule are both saturated. Pulmonary surfactant is represented by several different structural entities including the lamellar bodies, tubular myelin, and the monolayers of lipid and proteins. Lamellar bodies are dense multilayer structures formed in type II cells and secreted into alveolar space by exocytosis. The mechanism by which phospholipids are packaged into the lamellar body

is unknown, but ABCA transporters have been shown recently to be involved in transmembrane transport of endogenous lipids [3,4,11,12], so ABCA3 might well function as a transmembrane transporter of the phospholipid components of pulmonary surfactant.

Shuman's group has generated monoclonal antibodies against the lamellar body membrane fraction of type II cells prepared from rat lung. One of them (Mab 3C9) recognizes specifically a 180-kDa integral membrane protein occurring primarily on the lamellar body [19]. In addition, they reported very recently that ABCA3 mRNA is abundant in type II cells and that its expression is induced highly during fetal lung differentiation [20]. Whether or not the Mab 3C9 antibody recognizes the ABCA3 protein is an intriguing question; although the molecular size of the ABCA3 protein detected by our antibody is different from that detected by the Mab 3C9 antibody, this might reflect species difference.

We have shown recently that ABCA2 is expressed exclusively in the oligodendrocytes in brain [17]. Oligodendrocytes produce the lamellar structure myelin, which is composed of phospholipids and specific myelin proteins. ABCA2 immunoreactivity is detected mostly around lysosomes [17]. Interestingly, the lamellar body of type II cells shares membrane proteins with lysosomes [21,22]. These results suggest that the structurally related ABCA2 and ABCA3 both are associated with the function of lysosomes. Further studies to clarify the functional roles of ABCA3 are needed.

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