Functional and Trafficking Defects in ATP Binding Cassette A3 Mutants Associated with Respiratory Distress Syndrome

Members of the ATP binding cassette (ABC) superfamily actively transport a wide range of substrates across cell and intracellular membranes. Mutations in ABCA3, a member of the ABCA subfamily with unknown function, lead to fatal respiratory distress syndrome (RDS) in the newborn. Using cultured human lung cells, we found that recombinant wild-type hABCA3 localized to membranes of both lysosomes and lamellar bodies, which are the intracellular storage organelles for surfactant. In contrast, hABCA3 with mutations linked to RDS failed to target to lysosomes and remained in the endoplasmic reticulum as unassembled forms. Treatment of those cells with the chemical chaperone sodium 4-phenylbutyrate could partially restore trafficking of mutant ABCA3 to lamellar body-like structures. Expression of recombinant ABCA3 in nonhuman lung embryonic kidney 293 cells induced formation of lamellar body-like vesicles that contained lipids. Small interfering RNA knockdown of endogenous hABCA3 in differentiating human ATII cells resulted in abnormal, lamellar bodies comparable with those observed in vivo with mutant ABCA3. Silencing of ABCA3 expression also reduced vesicular uptake of surfactant lipids phosphatidylcholine, sphingomyelin, and cholesterol but not phosphatidylethanolamine. We conclude that ABCA3 is required for lysosomal loading of phosphatidylcholine and conversion of lysosomes to lamellar body-like structures.

ATP binding cassette (ABC) transporters are a superfamily of highly conserved membrane proteins that transport a wide variety of substrates across cell membranes (1). Among the several subfamilies, the ABCA subclass has received considerable attention, because mutations of the ABCA1 gene cause Tangier disease and mutations of the ABCA4 gene cause Stargardt macular dystrophy in humans (2–5). ABCA1 and ABCA4 are proposed to be transmembrane transporters for intracellular cholesterol/phospholipids and N-retinylidene phosphatidylethanolamine, respectively (3–5). ABCA3, a member of the ABCA subfamily with unknown function (6–10), is predominantly expressed in the lung and localized to the limiting membrane of lamellar bodies in alveolar epithelial type II cells (ATII) in both humans and rats (7, 8).

In the lung, development of structures for effective pulmonary gas exchange and production of pulmonary surfactant are necessary for successful adaptation to extraterrestrial life in the newborn infant. These key processes in lung maturation require differentiation of epithelium into ATII cells, the cellular source for surfactant. Pulmonary surfactant is a complex mixture of lipids, primarily phosphatidylcholine (60–70% of which is dipalmitylophosphatidylcholine) and specific proteins that line the alveolar surface of the lung, reducing surface tension at the air-liquid interface and preventing collapse of the lung on expiration (11). Surfactant is assembled and stored in lamellar bodies, the secretary organelles of ATII cells (11–13). Two other members of the ABCA subfamily, ABCA1 and ABCA4, have been implicated in lipid transport leading to the hypothesis that ABCA3 transports lipid into the lamellar bodies of ATII cells (7–9). Recently, it has been reported that mutations in ABCA3 are associated with defective assembly of lamellar bodies and fatal respiratory distress syndrome (RDS) in the newborn infant and interstitial lung disease (6, 10).

To study the potential role of ABCA3 in RDS, we examined the subcellular trafficking and substrate specificity of ABCA3 in hATII cells and mammalian cell lines using green fluorescent protein (GFP)-tagged protein and fluorescent lipid analogs. Morphological and functional changes secondary to both loss- and gain-of-function experiments demonstrate that ABCA3 selectively transports phosphatidylcholine, sphingomyelin, and cholesterol to lamellar bodies in hATII cells. Our findings indicate that lipid trafficking by ABCA3 across lamellar body membranes is necessary for lamellar body biogenesis as a key step in assembly of lung surfactant in hATII cells.

MATERIALS AND METHODS

Reagents—PNGase F was obtained from New England Biolabs (Beverly, MA). Sodium 4-phenylbutyrate (SBP11) was purchased from Scandinavian Formulas, (Sellersville, PA). LysoTracker Red, ETRacker Red, and Nile-Red were obtained from Molecular Probes. Rabbit anti-surfactant protein B (SP-B) and mouse anti-actin antibodies were purchased from Chemicon International. Mouse anti-GFP antibody was purchased from BD Biosciences. DC-LAMP antibody was purchased from Immunotech (Beckman Coulter, Inc.). The LAMP-1 (H4A3) and LAMP-2 (H4B4) monoclonal antibodies developed by J. T. August and J. E. K. Hildreth were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA. C12-NBD-phosphatidylcholine, C12-NBD-sphingomyelin, C12-NBD-phosphatidylethanolamine, and NBD-cholesterol were purchased from Avanti Polar Lipids, and other lipids were purchased from Sigma. All

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other reagents were electrophoretic grade and obtained from either Sigma or Invitrogen.

**DNA Construct**—For hABCA3-GFP or hABCA3-DsRed construction, a DNA fragment containing a full-length hABCA3 construct was generated using the PCR method with 5'–XhoI primer (CTCGAGC-GATGGCTGTCGTCAAGGAG) and 3'–EcoRI primer (GAATTC-CTGTCGCTTCCTCTGTC). The PCR fragments were cloned to XhoI-EcoRI-digested pEGFP-N1 or pDsRed-N1 (Clontech) vectors. hABCA3 missense mutants (L101P, N568D, and G1221S) were generated using the PCR method with the following primers: L101P (forward primer, 5’–AGACAGTGGCAGAGCCACTGTTGATACACATGGCAGGAG–3’; reverse primer, 5’–CTCGGATGTGATCAAGGTGGCCTGGCAGCTGTCTC–3’); N568D (forward primer, 5’–ATCACCGTCTGCTGGGCCCAGTCGTTGGGAGG–3’; reverse primer, 5’–GTCTTCCTCCTCTTCAGACCGGGAGCAGCTGGAGATG–3’); and G1221S (forward primer, 5’–ATCTTCAACATCTGTGACGCCATCACAACCACATTCTCCTG–3’; reverse primer, 5’–CAGAGGAGTGGCCTGGCAGGTCAGGTTGAGATG–3’), where the mutated nucleotides are underlined. The PCR fragments were constructed using QuikChange II XL site-directed mutagenesis Kit (Stratagene).

**Mammalian Cell Lines, Culture, and Transfection**—Mammalian cell culture and transfection were performed as described previously (8). hABCA3-GFP/HEK293, GFP/HEK293, L101P-hABCA3-GFP/HEK293, and G1221S-hABCA3-GFP/HEK293 stable cell lines were selected for XhoI-EcoRI-digested pEGFP-N1 or pDsRed-N1 (Clontech) vectors. L101P and G1221S (forward primer, 5’–ATCTTCAACATCTGTGACGCCATCACAACCACATTCTCCTG–3’; reverse primer, 5’–CAGAGGAGTGGCCTGGCAGGTCAGGTTGAGATG–3’), and N568D (forward primer, 5’–ATCACCGTCTGCTGGGCCCAGTCGTTGGGAGG–3’; reverse primer, 5’–GTCTTCCTCCTCTTCAGACCGGGAGCAGCTGGAGATG–3’). The PCR fragment was subcloned into XhoI-EcoRI-digested pEGFP-N1 or pDsRed-N1 (Clontech) vectors. The PCR product was then purified and subcloned using the QuikChange II XL site-directed mutagenesis Kit (Stratagene).

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Real-time RT-PCR—Total RNAs were extracted from untransfected or siRNA-transfected hATII cells using the RNeasy Mini Kit (Qiagen) following the manufacturer’s instruction, and on-column DNase digestion was performed using RNase-free DNase (Qiagen) to remove trace genomic DNA. The yield and purity of RNA was spectrophotometrically determined. Real-time RT-PCR was performed on a LightCycler (Roche Applied Science) using a one-step LightCycler-RNA Master SYBR Green I Kit (Roche). The cycling condition for RT-PCR was as follows: 48 °C for 30 s, 95 °C for 10 s, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Quantification of the target gene mRNA level was obtained as a threshold PCR cycle number (Ct) when the increase in the fluorescent signal of the PCR product showed exponential amplification. This value was then normalized to the threshold PCR cycle number obtained for actin mRNA from a parallel sample. Real-time RT-PCR was performed using the following primers: hABCA3 (forward, 5’–TTCTTACCTACATCCCCCTAC–3’; reverse, 5’–CCTTTCGCCAATCCCCCTAC–3’). The hABCA3-GFP plasmid was electroporated into human fetal lung epithelial cells (human fetal epithelial cells) according to the manufacturer’s protocol (Nucleofector, Amaxa Biosystem GmbH, Cologne, Germany). Once nucleasefected, the cells were transferred into fresh Waymouth’s medium containing 10% fetal calf serum for attachment. 24 h post-nucleasefection, medium was changed to serum-free Waymouth’s medium containing DCl and cells cultured for an additional 4 days (15).

**Lipid Uptake**—NBD-lipid in membrane fractions was measured as previously described (18). Briefly, cells were incubated with NBD-lipid containing liposomes for 30 min and washed with ice-cold phosphate-buffered saline two times each for 5 min. Equal numbers of cells (1 × 10^7 cells) were permeabilized with 1 ml of intracellular medium composed of 120 mM KCl, 10 mM NaCl, 1 mM KH2PO4, 20 mM Tris-HEPES, and 1 µg/ml each aprotinin, leupeptin, and pepstatin, at pH 7.2, supplemented with 80 µg/ml digitonin (Sigma, 50% [w/v]). After 10 min of incubation, the intracellular medium was removed, and the cells were resuspended in 1 ml of phosphate-buffered saline. Fluorescence from the suspension was monitored in a multichannel excitation dual wavelength emission fluorometer (Delta RAM, Photon Technology International) using 460-nm excitation and 534-nm emission. Experiments were performed at 37 °C with constant stirring. Data are representative of four independent experiments.

**Electron Microscopy**—For electron microscopy, cells cultured on glass slides were fixed with ice-cold 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h, followed by two steps of post-fixation: 1% OsO4 in cacodylate buffer and then 2% uranyl acetate in distilled water.
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FIGURE 1. Targeting of hABCA3 to the membrane of the lamellar body and lysosomes. Human ATII cells differentiated with DCI treatment in vitro (a–f) and A549 cells (g–o) were transfected with hABCA3-GFP and stained with LysoTracker Red (a–c, g–i) or immunostained with DC-LAMP (d–f), LAMP-1 (j–l), or LAMP-2 (m–o) antibodies. In all of the merged confocal microscopy images (c, f, i, l, o), hABCA3-GFP consistently localized to vesicles and vesicle membranes (insets) containing lysosomal markers. Scale bar represents 10 μm.

RESULTS

hABCA3 Localizes to Lamellar Bodies and Lysosomes—ABCA3 was previously shown to localize to lamellar body membranes in ATII cells and to intracellular vesicles in lung-derived cell lines (7, 8, 19). Many secretory organelles appear to be derived from or consist of terminal lysosomes (20–24). We therefore hypothesized that lamellar bodies are lysosome-derived secretory organelles. hABCA3 fused to green fluorescent protein (hABCA3-GFP) was transiently expressed in A549 and hATII cells. In hormone-induced, differentiated human fetal lung ATII cells, which produce and secrete surfactant comparable with adult ATII cells, confocal microscopy showed hABCA3-GFP in the membranes of vesicles that were labeled with the lysosomal marker LysoTracker Red and the lamellar body membrane marker dendritic cells and vesicle membranes (DC-LAMP) (Fig. 1, a–f) (25). In A549 cells, a lung-derived epithelial tumor cell line that neither contains lamellar bodies nor expresses DC-LAMP and surfactant proteins (26), hABCA3-GFP, was found in membranes of vesicles that were labeled with LysoTracker Red and two lysosomal membrane proteins, LAMP-1 and LAMP-2 (Fig. 1, g–o). These results further support a close relationship between lysosomes and lamellar bodies in ATII cells.

hABCA3 Mutations Associated with RDS Alter Trafficking and Processing of the Protein—Missense mutations of ABCA3 linked to fatal surfactant deficiency and abnormal lamellar body formation have been found in different functional domains of hABCA3 (6, 10). To determine whether these mutations lead to improper organelle targeting or loss of ATPase activity, constructs of hABCA3-GFP were engineered with representative missense mutations in residues conserved across species for ABCA3 (6). The first was in extracellular loop 1 (L101P), the second in the Walker A motif or P-loop (phosphate binding loop) of the N-terminal nucleotide binding domain (N568D), and the third in transmembrane domain 11 (G1221S) of hABCA3. These three mutant ABCA3 constructs (shown schematically in supplemental Fig. 1) were then expressed in A549 cells (supplemental Fig. 2). Fluorescence images of live cells stained with either LysoTracker Red or ERTracker Red indicated that the three constructs had graded trafficking defects. The construct containing mutation L101P of ABCA3-GFP failed to target the lysosomal membrane (Fig. 2A, d–f) and mainly remained in the ER (Fig. 2B, d–f). The construct containing mutation G1221S occasionally localized to lysosomes (Fig. 2A, j–l, arrows) and otherwise remained in the ER (Fig. 2B, j–l). The construct containing mutation N568D often localized to the lysosomal membrane (Fig. 2A, g–i) and partially remained in the ER (Fig. 2B, g–i). Thus, single missense mutations of hABCA3 can alter its localization, suggesting that RDS in some affected infants is likely associated with improper intracellular trafficking of hABCA3.

Western blotting with GFP antibody revealed that mutant protein is expressed at a lower overall level than wild-type protein and that wild-type and mutant fusion proteins (L101P, N568D, and G1221S) are processed differently (Fig. 2C). hABCA3 protein was found as two molecular mass forms by SDS-PAGE, a 190-kDa form (GFP fusion protein = 220 kDa) and a 150-kDa form (GFP fusion protein = 180 kDa) (7). However, the relative amount of the lower molecular mass protein band was markedly reduced in all of the mutants compared with wild type. Densitometry analysis of a 180/220-kDa ratio of Western blot (Fig. 2C) was 0.85 for the wild-type protein, 0.45 for the N568D mutant, 0.3 for the G1221S mutant, and essentially 0.0 for the L101P mutant. The higher molecular mass (full-length) bands (220 kDa) for wild-type and mutant proteins were shifted by ~10 kDa to lower molecular masses after treatment with PNGase F (Fig. 2C, 210 kDa), whereas the positions of the lower molecular mass bands (180 kDa) were not affected by glycosidase, as shown previously for the wild-type protein (9).

Effect of 4-PBA on Trafficking of hABCA3 Missense Mutations—Improper protein folding or trafficking is associated with a number of genetic diseases (27, 28). Chemical or pharmacological chaperones can subsequently correct abnormal folding or trafficking of defective proteins (29, 30). Abnormal trafficking of the most common mutation of the cystic fibrosis gene (ABCC7), ΔF508-CFTR (cystic fibrosis transmembrane regulator), can be rescued by application of the chemical chaperone 4-phenylbutyrate (4-PBA) (31). To
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FIGURE 2. Subcellular localization and processing of ABCA3 with missense mutations related to RDS. LysoTracker Red (A) and ERTracker Red (B) staining of wild-type (a–c) and missense mutations of hABCA3 (L101P (d–f), N568D (g–i), and G1221S (j–l)) linked to GFP in transfected A549 cells. Scale bars represent 10 μm. C, missense mutations affected protein processing but not glycosylation of hABCA3. The wild-type, L101P, N568D, and G1221S hABCA3-GFP membrane fractions were incubated in the absence (−) or presence (+) of 250 units of PNGase F at 37 °C for 1 h. These samples were Western blotted with an antibody to GFP (upper panel) or β-actin (lower panel). hABCA3-GFP and β-actin bands are indicated by the arrows.
investigate whether trafficking of mutant hABCA3 could be restored, 4-PBA was applied to cells expressing the hABCA3-GFP missense mutants L101P hABCA3-GFP, and G1221S hABCA3-GFP and visualized by confocal microscopy. The addition of 1 mM 4-PBA to HEK239 cells stably transfected with G1221S hABCA3-GFP and L101P hABCA3-GFP markedly altered GFP localization from the ER to the membranes of punctate vesicles (Fig. 3, A and B, and supplemental Fig. 3), but it did not alter the localization of wild-type hABCA3-GFP (supplemental Fig. 4). Vesicles with G1221S hABCA3-GFP (Fig. 3 B, b, d, and f), but not L101P hABCA3-GFP (Fig. 3 A, b, d, and f), stained positively with LysoTracker Red. Western blots with GFP antibody showed that total hABCA3-GFP protein was increased in the presence of 4-PBA for both L101P and G1221S hABCA3-GFP (Fig. 3 C) but that the ratio of 180/220 kDa protein bands increased only for the G1221S hABCA3-GFP mutant (Fig. 3 D), providing further evidence that the lower molecular weight protein form correlates with lysosomal processing.

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**ABCA3 Promotes Formation of Lamellar Vesicles**—Mutations of hABCA3 protein are associated with abnormal lamellar body formation in hATII cells (6). To test whether ABCA3 is sufficient for biogenesis of vesicles containing lamellae, stable hABCA3-GFP expression was established in non-lung-derived HEK293 cells that normally express low levels of endogenous ABCA3. As with the other cells, hABCA3-GFP was localized to lysosomal membranes in HEK293 cells (supplemental Fig. 5). To examine the effects of ABCA3 expression on cell function and morphology, GFP/HEK293 or hABCA3-GFP/HEK293 cells were stained with Nile-Red, a lipophilic dye used to label lamellar bodies in hATII cells (15). Cells expressing hABCA3-GFP exhibited more vesicles that were stained with Nile-Red compared with GFP/HEK293 cells (Fig. 4 A), suggesting that ABCA3 expression may promote formation of lipid-containing vesicles. Ultrastructural visualization by electron microscopy of hABCA3-GFP/HEK293 cells stained with osmium tetroxide revealed intracellular vesicles with multilamellar structures composed of a dense lipid core (Fig. 4 B, a and b, arrows) in contrast to GFP/HEK293 cells (Fig. 4 C, c and d). This result corroborates earlier evidence that ABCA3 promotes the formation of lamellar body-like structures in non-lung-derived cell lines (9).

**Effect of ABCA3 Gene Silencing on Lamellar Body Biogenesis**—Differentiated human fetal lung ATII cells treated with hormones contain many lamellar bodies and express high levels of all surfactant proteins as well as ABCA3 (15). To further examine the role of hABCA3 in lamellar body biogenesis, hABCA3 mRNA was silenced using RNA interference in hATII cells. The increase in hABCA3 mRNA (relative to actin mRNA) in hormone-treated ATII cells was specifically and efficiently suppressed ~50% with 100 nM hABCA3 siRNA (Fig. 5 A). To determine the effect of hABCA3 suppression on lamellar body generation, we assessed the lamellar body-associated proteins SP-B and DC-LAMP by immunofluorescence. Following hABCA3 silencing, staining of large organelles with anti-SP-B and anti-DC-LAMP antibodies was markedly reduced in ATII cells (Fig. 5 B), indicating that ABCA3 protein expression is essential for lamellar body biogenesis. The lamellar bodies of hATII cells treated with ABCA3 siRNA were small and abnormal appearing (Fig. 5 C), similar to the ultrastructure of those observed in patients with fatal ABCA3 mutations.
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Silencing of ABCA3 Alters Lipid Uptake by Lamellar Bodies in hATII Cells—Our findings that ABCA3 expression is associated with the formation of organelles with lamellar body-like structure (Fig. 4 and Fig. 5, B and C) is consistent with previous suggestions that ABCA3 transports lipid into lamellar bodies (6–9). To directly test whether ABCA3 transports surfactant lipids and whether that transport is specific, we measured the uptake of a variety of lipids into the lamellar bodies of hATII cells. Earlier experiments showed that the fluorescent PC analog C12-NBD-phosphatidylcholine (C12-NBD-PC), when incorporated into liposomes, is rapidly taken up by lamellar bodies of ATII cells (16, 17).

We examined the role of ABCA3 in the uptake of fluorescent lipid to lamellar bodies under three differing conditions: (i) fetal alveolar epithelial cells were induced to differentiate into hATII cells with hormone treatment, (ii) hATII cells were differentiated with hormone and also treated with hABCA3 or nonspecific siRNAs, and (iii) human fetal alveolar epithelial cells without hormone were allowed to remain undifferentiated. All of the cells were then incubated with fluorescent NBD-tagged lipids and examined by fluorescence spectroscopy and confocal microscopy. Hormone treatment markedly increased both lamellar body uptake (Fig. 6A) and total cell membrane uptake (Fig. 6B) of C12-NBD-PC. The uptake of C12-NBD-PC was both qualitatively (Fig. 6A) and quantitatively (Fig. 6B) reduced (>55%) by ABCA3 siRNA treatment but not by nonspecific siRNA treatment. The uptake of C12-NBD-phosphatidylcholine (C12-NBD-SM) (supplemental Fig. 6A) and NBD-cholesterol (supplemental Fig. 6B) into LysoTracker Red-positive lamellar bodies in response to the treatments parallels that of C12-NBD-PC, whereas the uptake of C12-NBD-phosphatidylethanolamine (C12-NBD-PE) into lamellar bodies was unaffected by either specific or control siRNA (supplemental Fig. 6C).

Lipid Uptake by A549 Cells Expressing Wild-type and Mutant hABCA3-DsRed—To further characterize the influence of ABCA3 protein on lipid uptake, gain-of-function experiments were performed in A549 cells after transfection with hABCA3-DsRed to avoid GFP interference with the NBD fluorophore. Labeled lipids (C12-NBD-PC, C12-NBD-SM, NBD-cholesterol, and C12-NBD-PE) are taken up by both transfected cells (labeled with red) and untransfected cells (without red label) (Fig. 7A). The higher concentration regions of C12-NBD-PC and NBD-cholesterol were specifically localized to punctate DsRed-labeled vesicles (Fig. 7A, a–c and d–f). C12-NBD-SM (Fig. 7A, g–i) partially colocalized with hABCA3-DsRed-labeled vesicles in transfected A549 cells. Consistent with the hATII cell result, C12-NBD-PE did not colocalize with ABCA3-DsRed-labeled vesicles (Fig. 7A, j–l).

Because N568D and G1221S hABCA3-GFP protein partially localized to the lysosomal membrane, we examined whether those lysosomes would take up NBD-lipid. C12-NBD-PC did not colocalize with the N568D hABCA3-DsRed (Fig. 7B, a–c); however, a fraction of C12-NBD-PC colocalized with G1221S hABCA3-DsRed (Fig. 7B, d–f, arrows). Collectively, these results provide evidence that ABCA3 is essential for phosphatidylcholine uptake.

DISCUSSION

Lamellar bodies of ATII cells are intracellular storage sites for surfactant lipids that are essential for the stability of the alveoli and distal airways of the lung. Absence or reduction of surfactant leads to respiratory distress syndrome or reduction in breathing efficiency and gas exchange (12, 13). ABCA3 protein was identified as a lamellar body limiting membrane protein in ATII cells (7–9), and mutations in ABCA3 are associated with fatal lung surfactant deficiency in newborn infants (6, 10). In this study, we have demonstrated the role of ABCA3 in lamellar body biogenesis and lipid homeostasis in hATII cells.

Lamellar bodies contain lysosomal enzymes (e.g. acid phosphatase, cathepsin C and H) and lysosomal membrane proteins (LAMP-1, LAMP-2 and CD208), suggesting that they are of lysosomal origin and are modified for storage of newly synthesized material (20–24). This hypothesis is verified by the observation that hABCA3 protein, normally found only in lamellar body membranes, is trafficked to lysosomes in response to the treatments parallel that of C12-NBD-PC, whereas the uptake of C12-NBD-phosphatidylethanolamine (C12-NBD-PE) into lamellar bodies was unaffected by either specific or control siRNA (supplemental Fig. 6C).

To further characterize the influence of ABCA3 protein on lipid uptake, gain-of-function experiments were performed in A549 cells after transfection with hABCA3-DsRed to avoid GFP interference with the NBD fluorophore. Labeled lipids (C12-NBD-PC, C12-NBD-SM, NBD-cholesterol, and C12-NBD-PE) are taken up by both transfected cells (labeled with red) and untransfected cells (without red label) (Fig. 7A). The higher concentration regions of C12-NBD-PC and NBD-cholesterol were specifically localized to punctate DsRed-labeled vesicles (Fig. 7A, a–c and d–f). C12-NBD-SM (Fig. 7A, g–i) partially colocalized with hABCA3-DsRed-labeled vesicles in transfected A549 cells. Consistent with the hATII cell result, C12-NBD-PE did not colocalize with ABCA3-DsRed-labeled vesicles (Fig. 7A, j–l).

Because N568D and G1221S hABCA3-GFP protein partially localized to the lysosomal membrane, we examined whether those lysosomes would take up NBD-lipid. C12-NBD-PC did not colocalize with the N568D hABCA3-DsRed (Fig. 7B, a–c); however, a fraction of C12-NBD-PC colocalized with G1221S hABCA3-DsRed (Fig. 7B, d–f, arrows). Collectively, these results provide evidence that ABCA3 is essential for phosphatidylcholine uptake.
like vesicles, providing further evidence that hABCA3 is involved in the biogenesis of lamellar bodies from lysosomes (Fig. 4). This finding, along with a recent report from Nagata et al. (9), suggest hABCA3 transports bilayer-forming lipids into lysosomes. Although, hABCA3-transfected cells displayed large lipid-containing vesicles (Fig. 4A, Nile-Red) with some lamellae, the lamellae were less dense and not as well organized as in lamellar bodies of ATII cells (Fig. 4B). We speculate that the expression of hABCA3 alone is insufficient for the transition of lysosomes into distinct lamellar bodies. It is likely that other surfactant proteins, especially SP-B, may also be required for normal lamellar body biogenesis (32). Although not sufficient, ABCA3 appears necessary for lamellar body biogenesis. Ultrastructural visualization of differentiated ATII cells transfected with ABCA3 siRNA revealed immature and distorted lamellar body morphology (Fig. 5C) similar in structure to those observed in the lungs of newborns with RDS associated with ABCA3 mutations (6). In addition, these morphological changes coincide with the decrease of other proteins, such as SP-B and DC-LAMP in lamellar bodies (Fig. 5B).

**FIGURE 5.** Silencing of hABCA3 affects lamellar body biogenesis in hATII cells. A, endogenous hABCA3 mRNA in DCI-treated ATII cells is specifically suppressed 50% by ABCA3 siRNA. The quantity of mRNA measured with real-time RT-PCR was normalized to the corresponding levels of β-actin mRNA (bars represent S.D., n = 3). B, the localized immunofluorescence staining for both SP-B (a, c, e, g) and DC-LAMP (b, d, f, h) was suppressed by ABCA3-specific siRNA (e, f) but not by nonspecific siRNA (g, h). Scale bar represents 10 μm. C, lamellar bodies were large and contained many lamellae in electron micrographs of DCI-treated cells (+ DCI). They were smaller and less developed in the same cells treated with DCI plus ABCA3 siRNA (+ DCI, ABCA3 siRNA), similar to cells without DCI treatment (− DCI). Nonspecific siRNA did not affect lamellar body ultrastructure (+ DCI, Nonspecific siRNA). Scale bars represent 0.5 μm.
All three of the representative mutations tested from the currently identified group of nine missense mutations in ABCA3 that were associated with fatal surfactant deficiency (6) had impaired localization or function. The (L101P) mutant in extracellular loop 1 of ABCA3 had the most severe trafficking defect. The first extracellular loop of ABCA3 shares little sequence homology with other ABC transporters. However, several disease-causing missense mutations of human ABCA1 and ABCA4 in topologically related loops may also lead to mislocalization of the protein (33, 34). The (G1221S) mutant in transmembrane domain 11 of ABCA3 is somewhat localized to lysosomes and in some of those lysosomes it appeared to transport NBD-PC. This glycine is conserved in the 11th transmembrane domains of human ABCA1, A2, A3, A4, A7, and A12, but no disease-related mutations of this residue have been previously reported. The (N568D) mutant in the N-terminal P-loop of ABCA3 had the least severe trafficking defect but did not transport NBD-lipid. The crystal structures of isolated nucleotide binding domains from ABC transporters of bacterial or archael origin show the asparagine (or serine) from the P-loop coordinating the γ-phosphate of an ATP at the interface of a nucleotide binding domain dimer sandwich (35). Mutations in this residue might be expected to disrupt the ATP binding and the ATPase activity of ABCA3. Mutations of this asparagine in the N-terminal P-loop of ABCA1 and ABCA4 lead to Tangier disease and Stargardt macular dystrophy, respectively (36–38).

The failure of the mutants to traffic to lysosome was also associated with a substantial change in post-translational processing (Fig. 2C). Native ABCA3 protein from lung appears as either a 180- or 150-kDa band in Western blots, depending on species and/or antibody; however, both bands are present when exogenous ABCA3 is expressed in cell culture and the active form is uncertain (7–9). The ratio of amounts of low and high weight forms is lower for the mutants than the wild type. The ratio of 150- and 180-kDa protein for the G1221S mutant increases with 4-PBA treatment,
although it is not restored to the level of the wild-type protein. Whether or not the localization or processing defects are associated with the lethal phenotype is at present uncertain, as they appear to be coordinated. It may be that loss of carbohydrate residues along with the N-terminal portion of the protein directs or retains ABCA3 to membranes of lysosomes or lamellar bodies. The trafficking defect of hABCA3 missense mutants is likely because of improper protein folding and activation of the ER degradation pathway. In vivo folding of newly synthesized membrane proteins is dependent on chaperone molecules (e.g. heat shock proteins, calnexin) present in the ER (39–41). Recently, therapeutic approaches to restore function to misfolded ΔF508-CFTR used chemical (e.g. glycerol, Me₂SO) or pharmacologic (e.g. phenylbutyrate, flavonoids) chaperones to correct protein folding (31, 42). Our finding that 4-PBA treatment partially restored trafficking of G1221S hABCA3-GFP to the lysosomal membrane suggests that an approach using chemical or pharmacological chaperones should be explored further as a potential treatment for lung surfactant deficiency caused by ABCA3 mutations (Fig. 3B). However, we failed to observe a similar effect fol-
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Following 4-PBA treatment in the L101P mutant (Fig. 3d–f), suggesting that an additional protein(s) participates in the uptake of SM in ATII cells. NBD-labeled cholesterol uptake to lamellar bodies or lysosomes is also ABCA3-dependent (Fig. 7A, d–f, and supplemental Fig. 6B). A recent report that vanadate-induced 8-azido-[α32P]ATP trapping in ABCA3 requires cholesterol in the membrane (9) along with our NBD-cholesterol uptake results raise the possibility that ABCA3 may actively transport cholesterol into the lamellar body or lysosomal lumen. However, whether ABCA3 actively transports cholesterol specifically as well as choline-containing lipids or whether the lipid-containing vesicles act as a passive sink for cholesterol accumulation is uncertain and will require further biochemical analysis. In addition, it remains possible that ABCA3 may also participate in the transport of other surfactant phospholipids, such as phosphatidylserine, phosphatidylglycerol, or phosphatidylinositol.

In summary, we investigated the role of ABCA3 in lamellar body formation and the mechanism by which ABCA3 mutations in human neonates may cause surfactant deficiency. Our findings suggest that ABCA3 is essential for lamellar body formation, acting directly to promote the accumulation of phosphotidylcholine and indirectly promoting inclusion of proteins, such as SP-B, which is required for active surfactant. Further studies using purified hABCA3 protein are required to elucidate the detailed mechanism of ABCA3 transport of choline lipids.

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