Characterization and Classification of ATP-binding Cassette Transporter ABCA3 Mutants in Fatal Surfactant Deficiency*

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The ATP-binding cassette transporter ABCA3 is expressed predominantly at the limiting membrane of the lamellar bodies in lung alveolar type II cells. Recent study has shown that mutation of the ABCA3 gene causes fatal surfactant deficiency in newborns. In this study, we investigated in HEK293 cells the intracellular localization and N-glycosylation of the ABCA3 mutants so far identified in fatal surfactant deficiency patients. Green fluorescent protein-tagged L101P, L982P, L1553P, Q1591P, and Ins1518fs/ter1519 mutant proteins remained localized in the endoplasmic reticulum, and processing of oligosaccharide from high mannose type to complex type. Vanadate-induced nucleotide trapping and ATP-binding analyses showed that ATP hydrolysis activity was dramatically decreased in the N568D, G1221S, and L1580P mutants, accompanied by a moderate decrease in ATP binding in N568D and L1580P mutants but not in the G1221S mutant, compared with the wild-type ABCA3 protein. In addition, mutational analyses of the Gly-1221 residue in the 11th transmembrane segment and the Leu-1580 residue in the cytoplasmic tail, and homology modeling of nucleotide binding domain 2 demonstrate the significance of these residues for ATP hydrolysis and suggest a mechanism for impaired ATP hydrolysis in G1221S and L1580P mutants. Thus, surfactant deficiency because of ABCA3 gene mutation may be classified into two categories as follows: abnormal intracellular localization (type I) and normal intracellular localization with decreased ATP binding and/or ATP hydrolysis of the ABCA3 protein (type II). These distinct pathophysiologies may reflect both the severity and effective therapy for surfactant deficiency.

Pulmonary surfactant is a complex mixture of lipids and proteins that lowers surface tension at the air–liquid interface and prevents atelectasis (1–5). Surfactant synthesized in alveolar type II cells is stored in lamellar bodies and secreted into the alveolar space by exocytosis. The most abundant lipid in pulmonary surfactant is phosphatidylcholine, especially dipalmitylphosphatidylcholine, and the major proteins in surfactant are surfactant protein (SP)2-A, SP-B, SP-C, and SP-D. In the late term of fetal lung development, several enzymes involved in lipid synthesis as well as surfactant proteins are up-regulated in preparation for adaptation to air breathing (6–9). Insufficiency of surfactant in neonates causes respiratory distress syndrome.

Three causal genes for respiratory distress syndrome identified to date are those encoding SP-B, SP-C, and ABCA3 (10–12). SP-B is an amphipathic polypeptide that is stored together with lipids in lamellar bodies and secreted into the alveoli, and its interaction with the surface of membrane is essential for the formation and maintenance of surfactant (13, 14). SP-B deficiency in infants reduces the number of lamellar bodies in alveolar type II cells, leading to lethal respiratory distress syndrome (10, 15). SP-C is a single membrane-spanning hydrophobic peptide that is stored with SP-B and lipids in lamellar bodies for secretion into alveolar space, and its insertion into the lipid membrane is thought to enhance surfactant spreading and stability (14, 16–18). Mutations in gene encoding SP-C are associated with interstitial lung disease in neonates and children (11, 20).

The function of ABCA3, a member of the A subfamily of ATP-binding cassette (ABC) transporters, is still unknown. However, because ABCA3 is expressed predominantly at the limiting membrane of the lamellar bodies in lung alveolar type II cells (21, 22), and the temporal profile and glucocorticoid responsiveness of ABCA3 expression (23) are similar to those of surfactant, we proposed that ABCA3 may be involved in surfactant secretion. Recently, Shulenin et al. (12) identified various ABCA3 mutations in patients with fatal surfactant deficiency. Furthermore, very recently, ABCA3 mutations were reported in children with interstitial lung disease (24), indicating more roles of ABCA3 in the pathophysiology of lung disease than expected. Mutations identified in patients with fatal sur-

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‡ The abbreviations used are: SP, surfactant protein; ABC, ATP-binding cassette;endo H, endoglycosidase H; ER, endoplasmic reticulum; NBD, nucleotide binding domain; PNGase F, peptide N-glycosidase F; PNS, post-nuclear supernatant; TM, transmembrane segment; GFP, green fluorescent protein; PVDF, polyvinylidene difluoride; CFTR, cystic fibrosis transmembrane conductance regulator.
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In this study, to determine the pathophysiological role of these mutations in fatal surfactant deficiency, we characterized the subcellular localization, glycosylation, and ATP binding and ATP hydrolysis activities of GFP-tagged wild type and the eight ABCA3 mutants so far identified in fatal surfactant deficiency patients, expressed in cultured cells. Analyses of the ABCA3 mutants permit classification of fatal surfactant deficiency due to ABCA3 mutation into two categories.

EXPERIMENTAL PROCEDURES

DNA Construction—The coding region of human ABCA3 cDNA without termination codon was ligated into the EcoRI and BamHI site of pEGFPN1 (Clontech) to generate pEGFPN1-ABCA3 coding ABCA3 protein fused with enhanced GFP at the C terminus. Partial cDNA fragments containing various fatal surfactant deficiency mutations (L101P, N568D, L982P, G1221S, L1553P, L1580P, Q1591P, W1142X, and Ins1518fs) were generated with PCR methods and replaced with the corresponding fragment of pEGFPN1-ABCA3. Other site-directed mutant plasmids of Gly-1221 and Leu-1580 were similarly generated. The plasmid sequences were confirmed and used for transient transfection experiments.

The coding regions of wild-type ABCA3-GFP or its mutants were inserted into the EcoRI site of pCAGIpuro, an expression vector driven by a CAG promoter and containing the internal ribosomal entry site puromycin N-acetyltransferase gene cassette, to generate pCAGIpuro-ABCA3GFP and its mutant plasmids. These pCAGIpuro plasmid sequences were confirmed and used for stable transfection experiments.

Confocal Microscopy—HEK293 cells were grown at 37 °C under 5% CO2 in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal calf serum and penicillin/streptomycin. HEK293 cells (3 × 106) were seeded into 100-mm dishes. Single colonies were isolated, and the expression of the C terminus. Partial cDNA fragments containing various fatal surfactant deficiency mutations (L101P, N568D, L982P, G1221S, L1553P, L1580P, Q1591P, W1142X, and Ins1518fs) were generated with PCR methods and replaced with the corresponding fragment of pEGFPN1-ABCA3. Other site-directed mutant plasmids of Gly-1221 and Leu-1580 were similarly generated. The plasmid sequences were confirmed and used for transient transfection experiments.

Confocal Microscopy—HEK293 cells were grown at 37 °C under 5% CO2 in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal calf serum and penicillin/streptomycin. HEK293 cells (3 × 106) were seeded into 100-mm dishes. After 24 h, HEK293 cells were co-transfected with wild-type or mutant pEGFP plasmid, pDsRed2-ER, and pECFP-Golgi (333 ng each) using FuGENE transfection reagent (Roche Applied Science). The transfected cells were cultured for 48 h and fixed with 4% paraformaldehyde. The cells were viewed with a Zeiss confocal microscope LSM510-META. For immunostaining of multivesicular bodies and lamellar bodies, A549 cells expressing wild-type ABCA3-GFP or mutants were disrupted in 50 mM Tris-HCl buffer (pH 7.5) containing 250 mM sucrose and complete protease inhibitor mixture by N2, cavitation, followed by centrifugation at 1,000 × g for 10 min to obtain PNS. PNS was centrifuged at 20,000 × g for 30 min, and the membrane pellet was suspended in 10 mM Tris-HCl buffer (pH 7.5) containing 250 mM sucrose and 0.1 mM EGTA, and further centrifuged at 20,000 × g for 10 min to obtain 100,000 × g membrane fraction. A 20,000 × g membrane fraction (20–30 μg of protein) was incubated with 10 μM 8-azido-[α-32P]ATP, 2 mM ouabain, 0.1 mM EGTA, 3 mM MgCl2, and 40 mM Tris-HCl (pH 7.5) in a total volume of 12 μl for 10 min at 37 °C in the presence or absence of 0.4 mM orthovanadate, as described previously (26). The reaction was stopped by adding 400 μl of ice-cold 40 mM Tris-HCl buffer containing 0.1 mM EGTA and 1 mM MgCl2. The supernatant containing unbound ATP was removed from the membrane pellet after centrifugation (20,000 × g, 10 min, 2 °C), and the procedure was repeated once. The pellets were resuspended in 1 μl of Tris-HCl buffer containing 0.1 mM EGTA and 1 μM MgCl2 and irradiated for 10 min (at 254 nm, 8.2 milliwatts/cm2) on ice. The samples were then electrophoresed on a 5% SDS-polyacryl-
amide gel and transferred to a PVDF membrane (Millipore). The radioactivities of photoaffinity-labeled protein (total 220-kDa noncleaved form plus 180-kDa cleaved form) were quantified using FLA-5000 (Fujifilm). Radioactivities in the absence of orthovanadate were subtracted from radioactivities in the presence of orthovanadate and are represented as means ± H S.D. (n = 4) after normalization to the ABCA3-GFP protein (total 220-kDa noncleaved form plus 180-kDa cleaved form). Statistical analysis was performed as described above.

Binding of ABCA3-GFP and Mutants with 8-Azido-[γ-32P]ATP—A 20,000 × g membrane fraction (20–30 μg of protein) prepared as described above from HEK293 cells stably expressing wild-type ABCA3-GFP or mutants was incubated with 20 μM 8-azido-[γ-32P]ATP, 2 mM ouabain, 0.1 mM EGTA, 3 mM MgCl2, and 40 mM Tris-HCl (pH 7.5), in a total volume of 15 μl for 10 min at 0 °C (27, 28). Proteins were irradiated for 5 min (at 254 nm, 8.2 milliwatts/cm²) on ice and then solubilized in RIPA buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS, and 0.5% sodium deoxycholate) containing protease inhibitor mixture for 30 min at 4 °C. After centrifugation (20,000 × g, 20 min, 2 °C), proteins were immunoprecipitated from the supernatant with the anti-human ABCA3 antibody (26). Samples were electrophoresed on a 5% SDS-polyacrylamide gel and transferred to a PVDF membrane. The radioactivities of photoaffinity-labeled protein (total 220-kDa noncleaved form plus 180-kDa cleaved form) were quantified using BAS-2000 (Fujifilm). To confirm the expression level of ABCA3-GFP proteins, the membrane was further analyzed by immunoblotting using anti-GFP antibody. Data normalized to the ABCA3-GFP proteins (total 220-kDa noncleaved form plus 180-kDa cleaved form) are represented as means ± H S.D. (n = 3). Statistical analysis was performed as described above.
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A

A549
ABCA3-GFP

e GFP
f LAMP3
g DAPI
d Merge

HEK293
ABCA3-GFP

i GFP
j ER
k Golgi
l Merge

HEK293
ABCA3-GFP

B

a N568D
b LAMP3
c Merge
d G1221S
e LAMP3
f Merge

g L1580P
h LAMP3
i Merge

j L101P
k LAMP3
l Merge

C

a L101P
b ER
c Merge
d L982P
e ER
f Merge
g L1553P
h ER
i Merge

j Q1591P
k ER
l Merge

m Ins1518fs
n ER
o Merge

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RESULTS

Subcellular Localization of ABCA3-GFP and Its Mutants—
We have shown previously that ABCA3 is expressed predominantly at the limiting membrane of the lamellar bodies in lung alveolar type II cells (21). Subcellular localization of ABCA3 at the intracellular vesicle membrane may be important for the function of ABCA3. When human lung adenocarcinoma A549 cells were stably transfected with pCAGIpuRO-ABCA3-GFP (expression plasmid for ABCA3 fused to GFP at its C terminus), most of the GFP fluorescence showed a ring-like appearance (Fig. 2A, panel a). Fluorescence signals of LAMP3, a marker of multivesicular bodies or lamellar bodies (29, 30), were mainly detected at GFP fluorescence-positive vesicles (Fig. 2A, panels b and d), indicating that ABCA3-GFP mainly localizes at the limiting membrane of multivesicular bodies or lamellar bodies in A549 cells. Next, HEK293 cells were transiently transfected with pEGFPN1-ABCA3. Most of the GFP fluorescence was located at dot-like vesicles (Fig. 2A, panel e), and fluorescence signals of LAMP3 were mainly detected at the GFP fluorescence-positive vesicles (panels f and h) as in A549 cells. The HEK293 cells then were co-transfected with pEGFPN1-ABCA3, pDsRed2-ER, and pECFP-Golgi plasmids for fluorescent labeling of ABCA3, the endoplasmic reticulum (ER), and the Golgi apparatus, respectively. Most of the GFP fluorescence showed a dot-like appearance (Fig. 2A, panel i), and a few signals of GFP fluorescence were merged with DsRed2 fluorescence of the ER and cyan fluorescent protein fluorescence of the Golgi apparatus (Fig. 2A, panels j–l). These results indicate that ABCA3-GFP is mainly localized at the LAMP3-positive intracellular vesicle membrane in HEK293 cells, mimicking the sorting of ABCA3-GFP in alveolar type II cells and A549 cells.

To examine the effect of the mutations found in fatal surfactant deficiency patients on subcellular localization of ABCA3, wild-type and mutant ABCA3-GFP (seven missense mutations L101P, N568D, L982P, G1221S, L1553P, L1580P, and Q1591P, and one nonsense mutation, Ins1518fs) were transiently expressed in HEK293 cells (Fig. 1A). The N568D, G1221S, and L1580P mutant proteins were mainly localized to the intracellular vesicle membrane in HEK293 cells, whereas the L101P, L982P, L1553P, Q1591P, and Ins1518fs mutant proteins were mainly localized to the ER (data not shown), confirming defective intracellular sorting of L101P, L982P, L1553P, Q1591P, and Ins1518fs ABCA3 mutant proteins.

Glycosylation and Processing of ABCA3-GFP and Mutants—
To examine the relationship between subcellular localization and N-glycosylation of ABCA3 proteins, the total membrane fraction from transiently transfected HEK293 cells was analyzed by immunoblotting using anti-GFP antibody. Two bands at about 220 kDa (noncleaved form) and 180 kDa (cleaved form) (Fig. 3A) were detected in the wild-type ABCA3-GFP protein, consistent with our previous report that both the 190-kDa noncleaved form and the 150-kDa cleaved form were detected when human ABCA3 without the GFP tag was overexpressed in HEK293 cells (26). In the N568D, G1221S, and L1580P mutant proteins, which were mainly localized to the intracellular vesicle membrane, both the 220-kDa noncleaved form and the 180-kDa cleaved form were detected, similar to wild-type protein (Fig. 3A). In contrast, in the L101P, L982P, L1553P, and Q1591P mutant proteins, which were mainly localized at the ER, the amount of the 180-kDa cleaved form was considerably decreased, compared with that of wild-type protein, to an undetectable level (Fig. 3A). In the L982P, L1553P, and Q1591P mutant proteins, although the amount of 220-kDa noncleaved–form protein appears increased compared with that of wild-type protein in Fig. 3A, the total amount of ABCA3-GFP (220-kDa noncleaved form plus 180-kDa cleaved form) did not differ significantly among seven missense mutant proteins and the wild-type protein (n = 3, data not shown). The 4452insT mutation of the ABCA3 gene in fatal surfactant deficiency (12) causes a frameshift at Gly-1518 and introduces a Trp residue followed by a stop codon, the encoded Ins1518fs mutant protein lacking the C-terminal polypeptide containing the Walker B sequence of NBD-2 (Fig. 1A). In the Ins1518fs mutant protein, the level of the cleaved form (expected size of 150 kDa) also was considerably decreased to an undetectable level (Fig. 3A).

To examine the processing of oligosaccharides prior to proteolytic cleavage, the total membrane fraction prepared from transiently transfected HEK293 cells was treated with PNGase F or Endo H and analyzed by immunoblotting (Fig. 3, B and C). Endo H cleaves two proximal N-acetylgalactosamine residues of the high mannose-type sugar chains but not of the complex-type ones, whereas PNGase F cleaves both types. Treatment with PNGase F increased the electrophoretic mobility of 220-

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FIGURE 2. Subcellular localization of wild-type ABCA3-GFP and mutant proteins in cultured cells. A, panels a–d; A549 cells stably expressing wild-type ABCA3-GFP (panel a) were grown on glass cover slips and processed for immunofluorescence labeling of LAMP3 (panel b), a marker of lamellar bodies and multivesicular bodies. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (c). A merged image of panels a–c is shown in panel d. Panels e–h, HEK293 cells transiently expressing wild-type ABCA3-GFP (panel e) were processed for immunofluorescence labeling of LAMP3 (panel f). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (g). A merged image of panels e–g is shown in panel h. Panels i–l, HEK293 cells transiently co-expressing wild-type ABCA3-GFP (panel i), DsRed2-ER (panel j), and cyan fluorescent protein-Golgi (panel k) are shown. A merged image of panels i–k is shown in panel l. HEK293 cells transiently expressing mutant ABCA3-GFP proteins (panels a, d, g, and j) were processed for immunofluorescence labeling of LAMP3 (panels b, e, h, and k); N568D (panels a–c), G1221S (panels d–f), L1580P (panels g–i), and L101P (panels j–l). Merged images are shown in panels c, f, i, and l. C, HEK293 cells transiently co-expressing mutant ABCA3-GFP proteins (panels a, d, g, j, and m) and DsRed2-ER (panels b, e, h, k, and n) are shown: L101P (panels a–c), L982P (panels d–f), L1553P (panels g–i), Q1591P (panels j–l), and Ins1518fs (panels m–o). Merged images are shown in (panels c, f, i, l, and o). The scale bar represents 5 μm.
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**FIGURE 3.** Processing and glycosylation of wild-type ABCA3-GFP and mutant proteins in HEK293 cells. A, 10 μg of total membrane fractions from HEK293 cells transiently transfected with GFP, wild-type (Wt) ABCA3-GFP, or mutants was subjected to SDS-PAGE (5%), transferred to PVDF membranes, and analyzed by using anti-GFP monoclonal antibody. The position of noncleaved 220-kDa ABCA3-GFP protein (wild-type and missense mutants) and 190-kDa protein (nonsense Ins1518fs mutant) is indicated. The position of cleaved 180-kDa ABCA3-GFP proteins (wild-type and missense mutants) and 150-kDa protein (nonsense Ins1518fs mutant) is also indicated. B, 10 μg of total membrane fraction with (+) or without (−) treatment of PNGase F was subjected to SDS-PAGE and immunoblot analysis. ABCA3-GFP proteins modified with oligosaccharide (220 kDa in wild-type and missense mutants and 210-kDa protein except for 190-kDa Ins1518fs protein) were deglycosylated by PNGase F and produced 210- and 180-kDa proteins (band II), respectively, indicating that all eight mutants are N-glycosylated as is wild-type ABCA3-GFP protein.

When the total membrane fraction from cells expressing wild-type ABCA3-GFP was analyzed by Endo H digestion, the amounts of the 220-kDa undigested ABCA3-GFP protein (band I) and of the 210-kDa digested protein (band II) were comparable (Fig. 3C). This indicates that about 50% of the noncleaved 220-kDa wild-type ABCA3-GFP protein is modified with complex-type sugar chains that are insensitive to Endo H (Fig. 3, C and D). In the N568D, G1221S, and L1580P mutant proteins, about 30–40% of the 220-kDa protein remained as Endo H-insensitive complex-type protein (Fig. 3, C and D, band I), indicating that processing of oligosaccharide from high mannose type to complex type is largely preserved in these mutants. However, in the L101P, L982P, L1553P, Q1591P, and Ins1518fs mutant proteins, the levels of complex-type protein (band I) were dramatically decreased compared with that of wild-type protein (Fig. 3, C and D). More than 95% of the 220-kDa proteins of the four missense mutants and the 190-kDa protein of the Ins1518fs nonsense mutant was sensitive to Endo H digestion, producing 210- and 180-kDa proteins (band II), respectively, indicating that most of these ABCA3 mutant proteins are modified with high mannose-type sugar chains. These results indicate that the N568D, G1221S, and L1580P mutant proteins are mainly localized at the intracellular vesicle membrane accompanied by processing of oligosaccharide from high mannose type to complex type, whereas the four missense mutant (L101P, L982D, L1553P, and Q1591P) and one nonsense mutant (Ins1518fs) proteins remain localized at the ER, with impaired processing of oligosaccharide.

**ATP Hydrolysis of ABCA3-GFP and Mutants**—To investigate the mechanism of loss of function of the N568D, G1221S, and L1580P mutant proteins that are trafficked to intracellular vesicles accompanied by processing of sugar chains as is wild-type ABCA3 protein, we examined ATP hydrolysis of wild-type ABCA3-GFP and the mutant proteins. ABCA3 protein efficiently traps Mg-ADP in the presence of orthovanadate, an analog of phosphate, and forms a stable inhibitory intermediate during the ATP hydrolysis cycle (26). The intermediate can be specifically photoaffinity-labeled in the membrane after ATP hydrolysis when 8-azido-[α-32P]ATP is used as an ATP analog.

To examine vanadate-induced nucleotide trapping in ABCA3-GFP and the mutant proteins, HEK293 cells stably produced 210-kDa (wild-type and missense mutants) and 180-kDa (Ins1518fs) proteins (band II). D, percentage of ABCA3-GFP protein modified with complex-type sugar chains in noncleaved protein. The amount of each protein was quantified by measuring the density of the band. The level of wild-type and mutant ABCA3-GFP protein in band I (Endo H-insensitive 220-kDa protein except for 190-kDa Ins1518fs protein) is represented as a percentage of total band I plus band II (Endo H-sensitive 210-kDa protein except for 180-kDa Ins1518fs protein). *, p < 0.05; **, p < 0.005 versus wild type. N.S., not significant.
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expressing wild-type or mutant (N568D, G1221S, and L1580P) ABCA3-GFP fusion proteins were established. In the stably expressing cells, wild-type and the mutant ABCA3-GFP proteins were mainly localized to intracellular vesicle membrane as in transiently expressed cells (data not shown). Among 20,000 × g membrane fractions of the cells expressing wild-type ABCA3-GFP (Fig. 4A, lane 3) and 8-azido-[γ-32P]ATP (Fig. 4A, lane 3), and photoaffinity labeling was induced in the presence of orthovanadate (Fig. 4A, lane 4). Although we used ABCA3-GFP fusion protein in this study, these results are consistent with our previous observation using ABCA3 without GFP fusion (26). In the N568D, G1221S, and L1580P mutant proteins, vanadate-induced nucleotide trapping was significantly decreased to 12, 33, and 39% of that of the wild-type protein, respectively (Fig. 4, A, lanes 5–10, and C). These results indicate that ATP hydrolysis activity is severely impaired in these mutants.

To examine ATP hydrolysis of the mutant retained to the ER, cells stably expressing L101P mutant protein were established. L101P mutant protein was mainly localized to the ER, consistent with the result in transiently expressing cells (data not shown). The vanadate-induced nucleotide trapping also was significantly decreased compared with that of wild-type protein (Fig. 4B, lanes 3 and 4), indicating that ATP hydrolysis activity as well as intracellular trafficking is impaired in the L101P mutant protein.

ATP Binding of ABCA3-GFP and Mutants—To clarify the mechanism of loss of ATP hydrolysis activity of the N568D, G1221S, and L1580P mutant proteins, we examined ATP binding of wild-type ABCA3-GFP and the mutant proteins. Among the 20,000 × g membrane fractions of the cells expressing wild-type ABCA3-GFP, the 220-kDa (noncleaved form) and the 180-kDa (cleaved form) proteins were photoaffinity-labeled with 8-azido-[γ-32P]ATP (Fig. 5A). The membrane fraction of the untransfected HEK293 cells was not photoaffinity-labeled. The level of photoaffinity labeling of the G1221S mutant protein was reduced nucleotide trapping was significantly decreased to 12, 33, and 39% of that of the wild-type protein, respectively (Fig. 4, A, lanes 5–10, and C). These results indicate that ATP hydrolysis activity is severely impaired in these mutants. To examine ATP hydrolysis of the mutant retained to the ER, cells stably expressing L101P mutant protein were established. L101P mutant protein was mainly localized to the ER, consistent with the result in transiently expressing cells (data not shown). The vanadate-induced nucleotide trapping also was significantly decreased compared with that of wild-type protein (Fig. 4B, lanes 3 and 4), indicating that ATP hydrolysis activity as well as intracellular trafficking is impaired in the L101P mutant protein.
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with 8-azido-[γ-32P]ATP was similar to that of wild-type ABCA3-GFP protein (Fig. 5, A and B). However, the levels of photoaffinity labeling of N568D and L1580P mutant proteins were moderately decreased to 60 and 54% of that of wild-type protein, respectively. The level of photoaffinity labeling of L101P protein remaining localized at the ER was considerably decreased to 25% that of wild-type protein. These results suggest that decreased ATP binding contributes to impaired ATP hydrolysis in the N568D, L1580P, and L101P mutants but not in the G1221S mutant.

ATP Hydrolysis of Site-directed Mutants of Gly-1221 in 11th Transmembrane Segment—Since transmembrane domains and NBDs are suggested to communicate during the ATP hydrolysis cycle (31), local environmental changes in the 11th transmembrane segment (TM-11) resulting from the G1221S mutation might allosterically impair ATP hydrolysis activity of the ABCA3 protein. To address this, the effects of introducing hydroxyl groups or alteration of side-chain size on ATP hydrolysis activity were investigated by generating three site-directed mutants (G1221A, G1221V, and G1221T), which were stably expressed in HEK293 cells. These mutant proteins were mainly localized to intracellular vesicle membrane (data not shown). In the G1221T mutant protein, which had hydroxyl-containing amino acids, vanadate-induced nucleotide trapping was decreased to 36% of that of wild-type protein, as also in G1221S mutant protein (Fig. 6, A, lanes 9–12, and B). In the G1221A and G1221V mutant proteins, which have a hydrophobic side chain, vanadate-induced nucleotide trapping was decreased to 15 and 18% of that of wild-type protein, respectively (Fig. 6, A, lanes 5–8, and B). This result indicates the significance of the small side chain of Gly-1221 (H atom) in TM-11 for ATP hydrolysis.

ATP Hydrolysis of Site-directed Mutants of Leu-1580 in NBD-2—Because both leucine and proline are hydrophobic amino acids, alteration of side-chain size could be responsible for the impaired ATP hydrolysis in the L1580P mutant. Accordingly, Leu-1580 was substituted with three hydrophobic amino acids, Ala, Val, and Phe, of different size. All three mutant proteins were stably expressed in HEK293 cells and were mainly localized to intracellular vesicle membrane (data not shown). Substitution with Val, the side chain of which is smaller than that of Leu, resulted in decreased vanadate-induced nucleotide trapping of 56% of that of wild-type protein (Fig. 7, A, lanes 7 and 8, and B). Substitution with Ala, the side chain of which is much smaller than that of Val, resulted in decreased vanadate-induced nucleotide trapping of 13% that of wild-type protein (Fig. 7, A, lanes 5 and 6, and B). On the other hand, substitution with Phe, the side chain of which is larger than that of Leu, also caused a dramatic decrease in vanadate-induced nucleotide trapping to 13% that of wild-type protein (Fig. 7, A, lanes 9 and 10, and B). These results indicate that appropriate side-chain size of Leu-1580 at NBD-2 is important for ATP hydrolysis of ABCA3.

Homology Modeling of NBD-2 of ABCA3—The relationship between side-chain size and ATP hydrolysis activity of Leu-1580 mutant proteins suggests side-chain contact of Leu-1580 with other amino acids in the ABCA3 protein. To address this question, the secondary structures of NBD of ABCA3 and other

![Figure 6. Vanadate-induced nucleotide trapping in site-directed mutant proteins of Gly-1221. A. 20,000 × g membrane fraction prepared from HEK293 cells stably expressing wild-type (WT) ABCA3-GFP (lanes 3 and 4), G1221A (lanes 5 and 6), G1221V (lanes 7 and 8), G1221T (lanes 9 and 10), or untransfected HEK293 cells (lanes 1 and 2) was incubated with 10 μM 8-azido-[γ-32P]ATP in the absence (−) or presence (+) of 0.4 mM orthovanadate (Vi) and 3 mM MgCl2 for 10 min at 37 °C. Proteins were photoaffinity-labeled with UV irradiation after removal of unbound ATP, electrophoresed on SDS-PAGE (5%), and transferred to a PVDF membrane. Membrane was analyzed by autoradiography (upper panel) and immunoblotting (β) using anti-GFP antibody (lower panel). B, radioactivities of photoaffinity-labeled protein (total 220-kDa noncleaved form plus 180-kDa cleaved form) were quantified by FLA-5000. Radioactivities in the absence of orthovanadate were subtracted from radioactivities in the presence of orthovanadate and are expressed after normalization to ABCA3-GFP protein (total 220-kDa noncleaved form plus 180-kDa cleaved form). Data are represented as means ± S.D. (n = 4–5), * p < 0.01 versus wild type. ABC transporters were first predicted (32) and compared with that of Escherichia coli maltose transporter MalK (Fig. 8A), of which the crystal structure has been solved (33). Leu-1580 was predicted to locate at helix 7 (referred from the study of vitamin B transporter BtuD; Ref. 34) adjacent to the H-loop His residue, which is well conserved and known to form a strong hydrogen bond with the γ-phosphate of ATP. We then modeled the structure of NBD-2 of ABCA3 based on the ATP-bound closed structure of MalK (Protein Data Bank entry 1Q12; Ref. 33) using SWISS-MODEL (35). In the model of ABCA3, the orientations of His-1572 and Leu-1580 in ABCA3 were similar to those of His-192 and Leu-200 in MalK, respectively (Fig. 8, B and C). In the model of ABCA3, Trp-1554 is the most proximal residue from Leu-1580 in amino acids located at helix 6, and the distance from δ-carbon of Leu-1580 to β-carbon of Trp-1554 (the nearest carbon) was ~4.4 Å, which is close to van der Waals contact. In MalK, the distance from δ-carbon of Leu-200 to β-carbon of corresponding Arg-173 at helix 6 was ~4.2 Å, comparable with the calculated distance in ABCA3. Substitution of Leu-1580 with Pro, considering the best rotamer conformation, extended the distance from γ-carbon of Pro-1580 to β-carbon of Trp-1554 to 5.4 Å (Fig. 8D). In the L1580V mutant protein,
which had moderately impaired ATP hydrolysis, the distance from \( \gamma \)-carbon of Val-1580 to \( \beta \)-carbon of Trp-1554 was calculated as 4.9 Å (Fig. 8F). In the L1580A and L1580F mutant proteins, which had dramatically impaired ATP hydrolysis, the distance from \( \beta \)-carbon of Ala-1580 and \( \zeta \)-carbon of Phe-1580 was extended to 6.3 Å and shortened to 2.2 Å, respectively, compared with that of wild-type protein (Fig. 8, E and G). These biochemical and modeling analyses suggest that an appropriate distance between Trp-1554 at helix 6 and 1580th amino acid at helix 7 in NBD-2 is important for ATP hydrolysis of the ABCA3 protein. Thus, impaired ATP hydrolysis in the L1580P mutant protein may result in part from the alteration of side-chain size.

### DISCUSSION

Pulmonary surfactant, composed mainly of phospholipids and specific surfactant proteins, reduces the surface tension at the alveolar air-liquid interface, thereby preventing the lungs from collapsing. However, the mechanisms of surfactant production in alveolar type II cells and secretion into the alveolar space are unknown. Recently, mutations in the ABCA3 gene were found in newborns with fatal surfactant deficiency (12). In this study, we examined the intracellular localization and N-glycosylation of eight ABCA3 mutant proteins, most of the mutations found in fatal surfactant deficiency to date. In addition, we examined ATP hydrolysis and ATP binding activities of the representative mutants.

Investigating the intracellular localization and N-glycosylation of these ABCA3 mutant proteins in HEK293 cells, we found the missense L101P, L982P, L1553P, Q1591P, and nonsense Ins1518fs mutant proteins to be predominantly localized at the ER, with impaired processing of oligosaccharide. W1142X mutant ABCA3 protein, another nonsense mutant reported in fatal surfactant deficiency (12), also was predominantly localized at the ER with impaired processing of oligosaccharide (data not shown).

Some mutations of ABC transporters associated with human disease have been reported to induce intracellular mislocalization of the protein. For example, R587W and Q597R mutations of ABCA1, which are found in Tangier disease patients with high density lipoprotein deficiency, appear to be impaired in intracellular trafficking and localized predominantly to the ER (36). One amino acid (Phe-508) deletion of the cystic fibrosis transmembrane conductance regulator (CFTR) hampers trafficking of protein from the ER to the plasma membrane (37), and proper folding of the CFTR protein is thought to be essential for the coat complex II-dependent export of the protein from the ER (38). Interestingly, a single amino acid is substituted with a proline residue in the four mutant ABCA3 proteins (L101P, L982P, L1553P, and Q1591P) that are retained at the ER. As three of these mutations (L101P, L982P, and L1553P) are located in the predicted \( \alpha \)-helical structure of the ABCA3 protein (32) and the proline residue is known to be helix breaker (39), its introduction into the ABCA3 protein might well disrupt the \( \alpha \)-helical structure and hamper proper folding and intracellular translocation. The large C-terminal deletion of the ABCA3 protein (Ins1518fs and W1142X) also might hamper this process. Indeed, patients with homozygous mutations of L101P, L1553P, and W1142X have been reported to die of surfactant deficiency during the neonatal period, and electron microscopic study of lung tissue from patients with homozygous L1553P and W1142X mutations revealed smaller lamellar bodies than those in normal lung tissue (12). These observations suggest that trafficking of ABCA3 protein from the ER to the intracellular LAMP3-positive vesicle is essential not only for the function of the ABCA3 protein but also for the maturation of lamellar bodies and alveolar surfactant metabolism.

In contrast, the N568D, G1221S, and L1580P mutant proteins were localized to intracellular vesicle membrane accompanied by processing of oligosaccharide from high mannose type to complex type as found in wild-type ABCA3 protein. However, vanadate-induced nucleotide trapping analysis revealed ATP hydrolysis activity to be significantly decreased in N568D, G1221S, and L1580P mutant ABCA3 proteins compared with wild type. Thus, the mechanism of surfactant deficiency because of ABCA3 gene mutation can be classified into two categories as follows: abnormal intracellular trafficking (type I) and decreased ATP hydrolysis activity with normal intracellular trafficking (type II). Although patients with

![Characterization and Classification of ABCA3 Mutants](image)

**FIGURE 7.** Vanadate-induced nucleotide trapping in site-directed mutant proteins of Leu-1580. A, 20,000 × g membrane fraction prepared from HEK293 cells stably expressing wild-type (Wt) ABCA3-GFP (lanes 3 and 4), L1580A (lanes 5 and 6), L1580V (lanes 7 and 8), L1580F (lanes 9 and 10), L1580P (lanes 11 and 12), or untransfected HEK293 cells (lanes 1 and 2) was incubated with 10 \( \mu \)M 8-azido-[\( ^{32} \)P]ATP in the absence (−) or presence (+) of 0.4 mM orthovanadate (V) and 3 mM MgCl\(_2\) for 10 min at 37 °C. Proteins were photoaffinity-labeled with UV irradiation after removal of unbound ATP, electrophoresed on SDS-PAGE (5%), and transferred to a PVDF membrane. Membrane was analyzed by autoradiography (upper panel) and immunoblotting (IB) using anti-GFP antibody (lower panel). B, radioactivities of photoaffinity-labeled protein (total 220-kDa noncleaved form plus 180-kDa cleaved form) were quantified by FLA-5000. Radioactivities in the absence of orthovanadate were subtracted from radioactivities recorded in the presence of orthovanadate and are expressed after normalization to ABCA3-GFP protein (total 220-kDa noncleaved form plus 180-kDa cleaved form). Data are represented as means ± S.D. (n = 3–4), *p < 0.01 versus wild type.

|   | HEK | Wt | L1580A | L1580V | L1580P |
|---|-----|----|--------|--------|--------|
| VI Lane | - | + | + | + | + |
| 1 Vanadate trap | - | + | + | + | + |
| 2  | + | + | + | + | + |
| 3  | + | + | + | + | + |
| 4  | + | + | + | + | + |
| 5  | + | + | + | + | + |
| 6  | + | + | + | + | + |
| 7  | + | + | + | + | + |
| 8  | + | + | + | + | + |
| 9  | + | + | + | + | + |
| 10 | + | + | + | + | + |
| 11 | + | + | + | + | + |
| 12 | + | + | + | + | + |
homozygous type II ABCA3 mutations have not been reported, patients with type I/type II compound heterozygous ABCA3 mutations (L982P/G1221S and Ins1518fs/L1580P) died of surfactant deficiency during the neonatal period, and the lamellar bodies of lung tissue from a patient with L982P/G1221S were reported to be smaller than those from normal lung tissue (12). Because type I ABCA3 mutation on one allele does not result in fatal surfactant deficiency, these results suggest that a sufficient level of ATP hydrolysis activity of the ABCA3 protein encoded in the other allele is essential for the function of the ABCA3 protein, maturation of the lamellar bodies, and surfactant metabolism.

Type II mutations lie in various locations as follows: N568D in the Walker A motif of NBD-1, G1221S in TM-11, and L1580P in NBD-2 (Fig. 1A). Because Asn-568 is highly conserved in the Walker B motif and is thought to be critical for the binding of γ-phosphate of ATP, as predicted...
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from the crystal structure of other ABC transporters (40), both ATP binding and ATP hydrolysis activities should be impaired in the N568D mutant protein.

It has been reported that some mutations in transmembrane domains of ABC transporters affect the activity of NBDs. For example, the E1204L mutation in TM-16 of MRP1 (multidrug resistance associated protein 1) affects vanadate-induced nucleotide trapping as well as transport activity of the protein (41). In this study, mutational analysis of Gly-1221 in ABCA3 has shown the significance of the side chain of the Gly residue (H atom) in TM-11 for ATP hydrolysis. In membrane proteins, Gly is found at twice the frequency than in soluble proteins, and it is suggested that the small side chain of Gly is important for tight helix packing and helix-helix association of membrane proteins in the lipid bilayer (42). Because conserved Gly residues were found in TM-5, TM-6, TM-10, TM-11, and TM-12 of the ABCA subfamily, and also in TMs of other subfamilies such as TM-17 in the ABCC subfamily (43), these Gly residues may well contribute to the geometry of TMs that is important for communication between transmembrane domains and NBDs.

Mutational analysis of Leu-1580 suggested that impaired ATP hydrolysis in the L1580P mutant protein is in part due to the change in side-chain size. However, because Pro is known as a helix breaker (39), disruption of helix 7 by the introduction of the Pro residue may also contribute to the impaired ATP hydrolysis in the case of the L1580P mutant protein. Homology modeling of NBD-2 of ABCA3 showed that the distance between 1580th amino acid acid at helix 7 and Trp-1554 at helix 6 of NBD-2 is related to ATP hydrolysis of the ABCA3 protein. Because helix 7 was adjacent to the H-loop His residue, it is possible that helix 7 interacts with helix 6 to maintain the orientation of His for efficient hydrogen bonding with the γ-phosphate of ATP. Although further confirmation of this interaction might be provided by mutational analysis of Trp-1554, many disease-related mutations at helix 6 and helix 7 of NBDs such as R2106C and E2131K in ABCA4 (44–47), F587I and L610S in ABCB7/CFT (48–50), and A665T in ABCB3/TAP2 (51) (Fig. 8A) support the importance of these helices for the function of the ABC transporter.

In this study, the 180-kDa cleaved protein in stably transfected cells appears to be higher in wild-type than in transiently transfected cells (Figs. 3–5). Indeed, the former is about 20–45% and the latter is about 10–20% of total 220-kDa noncleaved form plus the 180-kDa cleaved form, using total membrane fractions (data not shown). In addition, the level of vanadate-induced nucleotide trapping of the 180-kDa cleaved form protein is approximately two times higher than that of the 220-kDa noncleaved form protein after normalization to the protein level (Fig. 4A, lanes 3 and 4). Because cleaved protein is predominantly expressed in native lung tissue (21, 23), the processing of ABCA3 protein from noncleaved form to cleaved form may be physiologically important in ABCA3 function. Further studies are needed to understand the structure-function relationships of the ABCA3 protein.

We examined ATP binding and ATP hydrolysis of the type I mutant ABCA3 protein by using cells stably expressing L101P mutant protein, and we found that both ATP binding and ATP hydrolysis activities as well as intracellular trafficking were impaired in the L101P mutant protein. Although we did not examine ATP binding and ATP hydrolysis of other type I mutants, these results indicate that intracellular trafficking and also ATP binding and ATP hydrolysis activities are impaired in some type I mutant ABCA3 proteins.

Thus, intracellular trafficking and/or activity of ATP hydrolysis is dramatically impaired in the ABCA3 mutant proteins so far found in fatal surfactant deficiency patients. Such ABCA3 protein dysfunction could well underlie the severe phenotype of these patients. Very recently, it has been reported that the E292V mutation of the ABCA3 gene is responsible in the genetic etiology of pediatric interstitial lung disease related to abnormal surfactant function (24), the phenotype of which is milder than that of fatal surfactant deficiency. It is possible that the E292V mutation causes less severe disruption of intracellular trafficking or ATP hydrolysis activity. Additional biochemical studies are required to clarify the loss of function mechanisms for the mutations associated with milder pediatric interstitial lung disease.

Very recently, Cheong et al. (19) reported that L101P, G1221S, and N568D mutant proteins have the most severe, moderate, and the least severe trafficking and processing defects. In this study, the difference in degree of defect between N568D and G1221S mutant proteins is slight, if any. The reason for the difference between their results and ours is unknown, but it may be due to the different clonal cells used stably expressing ABCA3 mutants. With regard to the function of the ABCA3 mutant proteins, their findings indicating impaired colocalization of fluorescence-labeled phosphatidylcholine with ABCA3-positive vesicles in G1221S and N568D may well correlate with our findings indicating impaired ATP hydrolysis activities of these mutants.

In summary, the mechanisms of surfactant deficiency because of ABCA3 gene mutation can be classified into two categories, type I and type II, abnormal intracellular trafficking and decreased ATP hydrolysis activity. This distinction may be useful in assessing both the severity and effective therapy for lung disease because of mutation of the ABCA3 gene.

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