SR-BI binds both lipid-free SAA and SAA-containing HDL and also binds apoA-I and apoA-I-containing HDL (13). SAA molecules were cointernalized with transferrin (10) in an SR-BI-dependent manner (13). SAA binding to SR-BI-expressing cells caused activation of the ERK1/2 and p38 pathway (10), which was proposed to be a mechanism for SAA-mediated cytokine production (7). These reactions were blocked by HDL, apoA-I, and amphiphilic α-helical peptides (10). These findings, however, do not clearly help us to understand the roles of SAA in HDL metabolism.

Thus, the functions of SAA and SAA-HDL are still to be determined. They may be partly responsible for host defense mechanisms. They may help to remove cholesterol from an inflammation site. Finally, they may just be a secondary protein to the inflammatory event. Studying the production mechanism of SAA-containing HDL should help us to understand the functions of SAA.

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# Efflux of sphingomyelin, cholesterol, and phosphatidylcholine by ABCG1[S]

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**Abstract** **Cholesterol and phospholipids are essential to the body, but an excess of cholesterol or lipids is toxic and a risk factor for arteriosclerosis. ABCG1, one of the half-type ABC proteins, is thought to be involved in cholesterol homeostasis. To explore the role of ABCG1 in cholesterol homeostasis, we examined its subcellular localization and function. ABCG1 and ABCG1-K120M, a WalkerA lysine mutant, were localized to the plasma membrane in HEK293 cells stably expressing ABCG1 and formed a homodimer. A stable transformant expressing ABCG1 exhibited efflux of cholesterol and choline phospholipids in the presence of BSA, and the cholesterol efflux was enhanced by the presence of HDL, whereas cells expressing ABCG1-K120M did not, suggesting that ATP binding and/or hydrolysis is required for the efflux. Mass and TLC analyses revealed that ABCG1 and ABCA1 secrete several species of sphingomyelin (SM) and phosphatidylcholine (PC), and SMs were preferentially secreted by ABCG1, whereas PCs were preferentially secreted by ABCA1. These results suggest that ABCA1 and ABCG1 mediate the lipid efflux in different mechanisms, in which different species of phospholipids are secreted, and function coordinately in the removal of cholesterol and phospholipids from peripheral cells.**— Kobayashi, A., Y. Takanezawa, T. Hirata, Y. Shimizu, K. Misasa, N. Kioka, H. Arai, K. Ueda, and M. Matsuo. **Efflux of sphingomyelin, cholesterol, and phosphatidylcholine by ABCG1.** *J. Lipid Res.* **2006.** 47: **1791–1802.**

**Supplementary key words** ATP binding cassette protein G1 • ATP binding cassette protein A1 • high density lipoprotein

Cholesterol is important to the body as a component of cellular membranes and a precursor of steroid hormones. However, excess cholesterol is a risk factor for atherosclerosis. Thus, cholesterol levels are strictly regulated by synthesis and circulation in the body. Many ABC proteins are reported to function in lipid homeostasis (1). For example, ABCG5 and ABCG8 mediate the efflux of cholesterol and sitosterol from intestine and hepatocytes into intestinal lumen and bile duct (2, 3). ABCB4 (multidrug resistance 3) is a phosphatidylcholine (PC) flippase and functions in the secretion of PC into bile duct from hepatocytes (4). ABCA1 mediates the efflux of cholesterol and phospholipids from macrophages to form HDL (5).

ABCG1 is a half-type ABC protein, with a nucleotide binding fold (NBF) in its N-terminal half and a transmembrane region in its C-terminal half. Human *ABCG1* cDNA was cloned as a gene homologous to *white* in *Drosophila* (6), a transporter of eye pigments, and many N-terminal variant forms of ABCG1 have been reported (7, 8). Other members of the ABCG subfamily form a dimer to function. For example, ABCG2 forms a homodimer to participate in multidrug resistance in breast cancer cells (9), whereas ABCG5 and ABCG8 form a heterodimer to function in the efflux of sterol from cells in the small intestine and in hepatocytes (10, 11). Cserepes et al. (12) showed that a WalkerA lysine mutant of ABCG4 inhibited the ATPase activity of ABCG1 in a dominant-negative manner and suggested that ABCG1 can heterodimerize with ABCG4, which is most homologous to ABCG1 among the ABCG subfamily. Cross-linking experiments suggested that ABCG1 forms a homodimer (13). However, it has not been clearly shown that ABCG1 functions as a homodimer.

It is thought that ABCG1 is involved in transporting cholesterol, because it is induced upon the loading of macrophages with cholesterol via a pathway of nuclear hormone receptors, liver X receptor (LXR) and retinoid X receptor (RXR) (7, 14, 15). The *ABCG1* gene is expressed in lung, brain, spleen, and macrophages. In liver, ABCG1 is expressed mainly in Kupffer cells (16). Expression of

Abbreviations: apoA-I, apolipoprotein A-I; DSP, dithiobis (succinimidylpropionate); DTBP, dimethyl 3,3′-dithiobispropionimidate-HCl; LXR, liver X receptor; NBF, nucleotide binding fold; PC, phosphatidylcholine; RXR, retinoid X receptor; SM, sphingomyelin; sulfo-NHS-biotin, sulfo-*N*-hydroxysuccinimidobiotin.

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ABCG1 was increased in macrophages from patients with Tangier disease compared with control macrophages (17). Kennedy et al. (18) reported that disruption of *ABCG1* in mice on a high-fat, high-cholesterol diet showed accumulation of both neutral lipids and phospholipids in hepatocytes and macrophages, whereas overexpression of *ABCG1* protected murine tissues from lipid accumulation. Endogenous ABCG1 is reported to localize to the perinuclear region and, in some cases, is distributed in the plasma membrane of macrophage-derived foam cells (14, 17). It was reported that ABCG1 mediates the efflux of cholesterol from cells to HDL-2 or HDL-3 but not to lipid-poor apolipoprotein A-I (apoA-I) (19) and that ABCG1 redistributes cholesterol to cell surface domains accessible for removal by HDL (13). Consistent with these reports, inhibition of ABCG1 protein expression resulted in reduced HDL-3-dependent efflux of cholesterol and phospholipids in macrophages (14). These findings suggest that ABCG1 is involved in lipid efflux in peripheral cells, like ABCA1. However, the mechanism of efflux by ABCG1 is not clear.

In this study, we investigated the subcellular localization and function of ABCG1. We demonstrated that ABCG1 localized to the plasma membrane in a HEK293 stable cell line and that ABCG1 mediates the efflux of cholesterol and phospholipids [preferentially sphingomyelin (SM)] from cells.

## MATERIALS AND METHODS

### Materials

Rabbit polyclonal anti-ABCG1 antibody, goat polyclonal anti-ABCG1 antibody, mouse monoclonal anti-myc antibody, goat polyclonal anti-myc antibody, and mouse monoclonal anti-FLAG antibody were purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-ABCG8 antibody and mouse monoclonal anti-PentaHis antibody were obtained from Novus Biologicals and Qiagen, respectively. Rabbit polyclonal anti-FLAG and anti-BSA antibodies were purchased from Sigma. Mouse monoclonal anti-ABCA1 antibody was prepared as described previously (20). *ABCG5* and *ABCG8* cDNA were cloned from a human liver cDNA library. Dithiobis (succinimidylpropionate) (DSP), dimethyl 3,3′-dithiobispropionimidate-HCl (DTBP), and sulfo-*N*-hydroxysuccinimidobiotin (sulfo-NHS-biotin) were purchased from Pierce. Marathon cDNA libraries from fetal liver or placenta were obtained from Clontech. 8-Azido-[$\alpha$-$^{32}$P]ATP was purchased from Affinity Labeling Technologies. Other chemicals were purchased from Sigma, Amersham Biosciences, Wako Pure Chemical Industries, and Nacalai Tesque.

### Cloning of *ABCG1* cDNA

*ABCG1* cDNA was cloned from a human fetal liver and placental cDNA library. The cDNA sequence cloned in this study was the same as that reported by Kennedy et al. (7) (GenBank accession number NM_207630) except for its N terminus, which corresponded to a variant reported by Chen et al. (6) (GenBank accession number X91249). A WalkerA lysine mutant *ABCG1* (ABCG1-K120M) was prepared with the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) as described by the manufacturer. The cloned cDNA was inserted into the *Not* I site of pcDNA3.1(+) (Invitrogen) to make an expression vector for pcDNA3.1(+)/ABCG1 and pcDNA3.1(+)/ABCG1-K120M, or into the *NotI-XbaI* site of pcDNA3.1(+)A-mycHis (Invitrogen) or pcDNA3.1(+)A-FLAG [prepared by inserting FLAG tag sequences instead of myc and His tag sequences of pcDNA3.1(+)-A-mycHis] to make an expression vector for pcDNA3.1(+)A-mycHis/ABCG1 or pcDNA3.1(+)A-FLAG/ABCG1.

### Cell culture

HEK293 cells were grown in DMEM supplemented with 10% (v/v) FBS in 5% $CO_2$ at 37°C. THP-1 cells were grown in RPMI 1640 medium supplemented with 10% (v/v) FBS in 5% $CO_2$ at 37°C. The differentiation of THP-1 monocytes into macrophages was induced with 0.2 μg/ml phorbol 12-myristrate 13-acetate (Wako Pure Chemical) for 4 days. The differentiated cells were cultured in RPMI 1640 medium and 0.2% BSA for 24 h, and ABCG1 expression was induced for 24 h by adding TO901317 (Cayman).

### Establishment of a stable transformant of ABCG1

HEK293 cells were transfected with pcDNA3.1(+)/ABCG1 or pcDNA3.1(+)/ABCG1-K120M using LipofectAMINE (Invitrogen) according to the manufacturer's instructions. Cells were selected with 1 mg/ml geneticin (G418) for 2 weeks. Single colonies were isolated, and the expression of ABCG1 was examined by Western blotting and immunofluorescent staining with rabbit polyclonal anti-ABCG1 antibody.

### Glycosylation of ABCG1

Digestion with endoglycosidase H and peptide *N*-glycosidase F (New England Biolabs, Beverly, MA) was done as described by the manufacturer. In brief, 20 μg of membrane protein from cells was treated with 500 units of endoglycosidase H or 0.3 units of peptide *N*-glycosidase F for 1 h at 37°C. The deglycosylated proteins were electrophoresed on a 10% SDS-polyacrylamide gel and immunodetected using rabbit polyclonal anti-ABCG1 antibody.

### Immunostaining and fluorescence microscopy

Cells were cultured on glass cover slips, fixed with 4% paraformaldehyde in PBS$^+$ (phosphate-buffered saline containing 0.1 g/l $CaCl_2$ and $MgCl_26H_2O$), and permeabilized with 0.4% Triton X-100 in PBS$^+$ for 5 min. To diminish the nonspecific binding of antibodies, the cells were incubated in 10% goat serum in PBS$^+$. Cells were incubated for 1 h with rabbit polyclonal anti-ABCG1 antibody diluted 1:500 in PBS$^+$ containing 10% goat serum and then incubated with fluorescent Alexa 488-conjugated anti-rabbit IgG (Molecular Probes) for 1 h. Cells were directly viewed with a 63× Plan-Neofluar oil-immersion objective using a Zeiss confocal microscope (LSM5 Pascal).

### Biotinylation of cell surface proteins

Cells were washed with ice-cold PBS$^+$ and incubated with 0.5 mg/ml sulfo-NHS-biotin solubilized in PBS$^+$ for 30 min on ice in the dark. Cells were washed with PBS$^+$ to remove unbound sulfo-NHS-biotin and lysed in RIPA buffer [20 mM Tris-Cl (pH 7.5), 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate] containing protease inhibitors [100 μg/ml (*p*-amidinophenyl)-methanesulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml aprotinin). ImmunoPure Immobilized Monomeric Avidin Gel (Pierce) was added to the cell lysate to precipitate the biotinylated proteins. The biotinylated proteins were electrophoresed on a 10% SDS-polyacrylamide gel and immunodetected.

### Coimmunoprecipitation

After 48 h of transfection with pcDNA3.1(+)A-mycHis/ABCG1 or pcDNA3.1(+)A-FLAG/ABCG1, cells were washed with PBS and lysed in Nonidet P-40 lysis buffer [50 mM Tris-Cl (pH 7.5), 150 mM NaCl, and 1% Nonidet P-40] containing protease inhibitors. The lysates were incubated with antibodies and immunoprecipitated with protein G-Sepharose 4B Fast Flow (Sigma). The immunoprecipitated proteins were washed with Nonidet P-40 lysis buffer and electrophoresed on a 10% SDS-polyacrylamide gel.

### Cross-linking

Cells were washed with cold PBS and incubated with 250 μM DSP or DTBP at room temperature for 30 min. The cross-linking reaction was terminated by the addition of Tris-Cl buffer (pH 7.5) to 20 mM, and cells were incubated at room temperature for 15 min. Cells were washed with PBS and lysed in Nonidet P-40 lysis buffer. Samples were denatured in SDS sample buffer with or without DTT, electrophoresed on a 7% SDS-polyacrylamide gel, and immunodetected.

### Photoaffinity labeling

Membranes (20 μg of proteins) from HEK293 cells, prepared as described previously (21), were incubated with 50 μM 8-azido-[α-$^{32}$P]ATP in 3 μl of TEM buffer [40 mM Tris-Cl (pH 7.5), 0.1 mM EGTA, and 1 mM $MgCl_2$] containing 2 mM ouabain for 10 min on ice. Proteins were ultraviolet light-irradiated for 3 min (at 254 nm, 5.5 mW/cm$^2$) on ice. To remove free 8-azido-[α-$^{32}$P]ATP, ice-cold TEM buffer was added to the sample and the supernatant was removed after centrifugation (15,000 *g*, 5 min, 2°C). Pellets were resuspended in 100 μl of RIPA buffer containing 100 μg/ml (*p*-amidinophenyl)methanesulfonyl fluoride, and membrane proteins were solubilized for 30 min at 4°C. After centrifugation for 15 min at 15,000 *g*, the lysates were incubated with antibodies and immunoprecipitated with protein G-Sepharose 4B Fast Flow (Sigma). The immunoprecipitated proteins were washed with RIPA buffer, and samples were electrophoresed on a 10% SDS-polyacrylamide gel and autoradiographed.

### Fractional lipid release assay

Cells were subcultured on 12-well plates at a density of 5.0 × 10$^5$ cells. After incubation for 24 h, the cells were labeled with 2 μCi/ml [$^3$H]cholesterol or [$^3$H]choline for 24 h in DMEM containing 10% FBS. The cells were washed with fresh medium and incubated with DMEM containing 0.02% BSA in the absence or presence of 10 μg/ml apoA-I (Calbiochem) or 20 μg/ml HDL (Calbiochem) for 4 h. For cholesterol efflux, the medium was collected and the cells were lysed with 0.1 N NaOH and 0.1% SDS. For phospholipid efflux, phospholipids were extracted from the medium with chloroform-methanol (2:1) or from the cells with hexane-isopropanol (3:2). PC and SM were separated by TLC on silica gel 60 plates (Merck) developed in chloroform-methanol-acetic acid-water (60:30:10:5). The radioactivity was counted by liquid scintillation counting.

### Cellular lipid release assay

Cells were subcultured on six-well plates at a density of 1.0 × 10$^6$ cells. After incubation for 24 h, the cells were washed with fresh medium and incubated with DMEM containing 0.02% BSA in the absence or presence of 10 μg/ml apoA-I. The lipid content in the medium was determined after 24 or 48 h of incubation as described previously (22).

### Mass spectrometric analysis

Cells were subcultured on six-well plates at a density of 1.0 × 10$^6$ cells. After incubation for 24 h, the cells were washed with fresh medium and incubated with DMEM containing 0.02% BSA in the absence or presence of 10 μg/ml apoA-I for 48 h. The medium was centrifuged for 15 min at 7,000 *g* to remove cell debris twice. The lipids were extracted from 12 ml of medium by the method of Bligh and Dyer (23) after the addition of di-14:1 PC as an internal standard. The content of choline phospholipids in the medium was analyzed by MS. Mass spectrometric analyses were performed with a triple quadrupole instrument model Quattro micro (Micromass, Manchester, UK) equipped with an electrospray source as described previously (24). The samples were provided by the UltiMate high-performance liquid chromatography system (LC Packings, San Francisco, CA) into the electrospray interface at a flow rate of 4 μl/min in a solvent system of acetonitrile-methanol-water (2:3:1) containing 0.1% ammonium formate (pH 6.4). The mass spectrometer was operated in the positive and negative scan modes. The flow rate of the nitrogen drying gas was 12 l/min at 80°C. The capillary and cone voltages were set at 3.7 kV and 30 V, respectively, argon at 3–4 × 10$^{-4}$ Torr was used as the collision gas, and a collision energy of 30–40 V was used to obtain fragment ions for precursor ions. The relationship between peak height and amount of SM was examined using bovine brain SM (Avanti).

### Statistical analysis

Values are presented as means ± SD. Statistical significance was determined by Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

## RESULTS

### Expression of human ABCG1 protein in HEK293 cells

To analyze the subcellular localization and function of human ABCG1, the ABCG1 expression vector was introduced into HEK293 cells, and a cell line stably expressing ABCG1 (HEK/ABCG1) was established. HEK293 cells stably expressing ABCG1-K120M (HEK/ABCG1-K120M), a WalkerA lysine mutant, were also established. Both cell lines expressed similar amounts of ABCG1 migrating as ~60 kDa proteins on SDS-PAGE (**Fig. 1A**, lanes 4, 5). THP-1 cells, differentiated by phorbol 12-myristrate, faintly expressed ABCG1 migrating at ~60 kDa, and ABCG1 was induced by TO901317, a LXR ligand (Fig. 1A, lanes 1, 2). The amount of ABCG1 expressed in HEK/ABCG1 cells was comparable to that in THP-1 cells induced by TO901317. Other members of the ABCG subfamily (ABCG2, ABCG5, and ABCG8) have been reported to be glycosylated (10, 25). Indeed, ABCG5, expressed without ABCG8 and modified with high-mannose-type *N*-linked oligosaccharide, migrated faster after treatment with *N*-glycosidase F and endoglycosidase H (Fig. 1B, lanes 8–10), as reported (10). However, the migration of ABCG1, expressed in both THP-1 and HEK/ABCG1 cells, was not changed by treatment with either glycosidase (Fig. 1B, lanes 1–3, 5–7).

### Localization of ABCG1 in plasma membrane

ABCG1 was reported to be distributed mainly in the perinuclear region and only partly in the plasma mem-

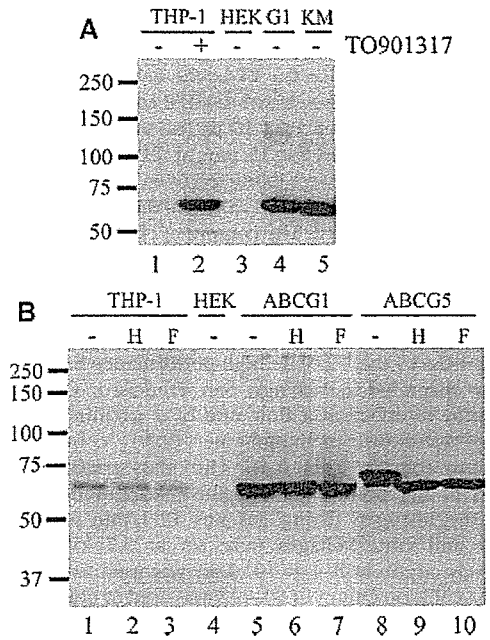


**Fig. 1.** Expression and glycosylation of ABCG1 in HEK293 and THP-1 cells. A: Cell lysates (10 μg of proteins) from THP-1 cells treated without (lane 1) or with 2.5 μM TO901317 (lane 2) or HEK293 cells (lane 3), HEK/ABCG1 cells (lane 4), or HEK/ABCG1-K120M cells (lane 5) were separated by 7% polyacrylamide gel electrophoresis. B: Membrane proteins (20 μg of proteins) from THP-1 cells treated with 1 μM TO901317 (lanes 1–3) or HEK293 cells (lane 4), HEK/ABCG1 cells (lanes 5–7), or HEK293 cells transiently expressing myc-tagged ABCG5 (lanes 8–10) were treated without (lanes 1, 4, 5, 8) or with endoglycosidase H (H; lanes 2, 6, 9) or peptide *N*-glycosidase F (F; lanes 3, 7, and 10). The samples were separated by 10% polyacrylamide gel electrophoresis. ABCG1 and ABCG5 were detected with rabbit polyclonal anti-ABCG1 or mouse monoclonal anti-myc antibody, respectively.

brane in macrophages (14, 17). However, immunostaining with anti-ABCG1 antibody suggested that the exogenously expressed ABCG1 was distributed mainly in the plasma membrane of HEK293 cells (**Fig. 2B, E**). No signal was detected in host HEK293 cells (Fig. 2A). ABCG1-K120M was also detected mainly in the plasma membrane (Fig. 2C, F), suggesting that the WalkerA lysine mutation did not affect the subcellular localization of ABCG1.

To confirm the cell surface expression of ABCG1, membrane proteins were biotinylated by sulfo-NHS-biotin and precipitated by avidin agarose (**Fig. 3**, upper panel). We also examined ABCA1 and ABCG8 as a positive and a negative control, respectively. ABCA1, which is localized mainly in the plasma membrane (20), was precipitated by avidin agarose after biotinylation (lane 2) but not without biotinylation (lane 1). On the other hand, ABCG8, which is distributed to the endoplasmic reticulum when expressed alone without ABCG5 (10), was not precipitated by avidin agarose (lane 4). ABCG1 and ABCG1-K120M were precipitated by avidin agarose after biotinylation (lanes 8, 10) but not without biotinylation (lanes 7, 9). These results indicate that ABCG1 localizes to the plasma membrane.

### Homodimerization of ABCG1

Half-type ABC proteins function as homodimers or heterodimers. ABCG2 localizes to the plasma membrane (25, 26) and functions as a homodimer (9), whereas ABCG5 and ABCG8 localize to the plasma membrane and function as heterodimers (10, 11). To examine whether ABCG1 forms a homodimer, ABCG1-myc and ABCG1-FLAG, in which each tag sequence was fused to the C terminus, were coexpressed, and immunoprecipitation was done with antibodies against tag sequences (**Fig. 4**, upper panel). When ABCG1-FLAG was immunoprecipitated with an anti-FLAG antibody, ABCG1-myc was coprecipitated (Fig. 4A, lane 4), and vice versa (Fig. 4B, lane 4). These results suggest that ABCG1 molecules interact with each other to form a homodimer or a homooligomer.

ABCG2 was reported to form a homodimer via a thiol bond (9) and to form homooligomers (27). The oligomeric features of ABCG1 were analyzed by chemical cross-linking with DSP and DTBP (**Fig. 5**). DSP and DTBP are cross-linking reagents with arm lengths of 12 and 11.9 Å, respectively, and both can be cleaved when reduced with thiols. When ABCG1 in living cells was cross-linked by DSP (lane 8) or DTBP (lane 14), ABCG1 with a molecular size of ~130 kDa was predominantly detected, which corresponds to the size of dimeric ABCG1, and was detected as a monomer when applied to SDS-PAGE after DTT treatment (lanes 11, 17). ABCG1 with higher molecular sizes was faintly detected. The electrophoretic mobility of ABCG1, without chemical cross-linking treatment on SDS-PAGE, was not altered when the sample was denatured in the absence of DTT (lane 2). ABCG1-K120M showed the same features of dimeric formation (lanes 9, 15) as the wild type. These results suggest that ABCG1 exists predominantly as a dimer without forming a thiol bond and that the WalkerA lysine mutation does not affect the dimeric formation of ABCG1.

### Binding of ATP by ABCG1

ABC proteins bind nucleotides at their NBFs and transport substrates using the energy of ATP hydrolysis. The binding of ATP by ABCG1 was analyzed using a photoaffinity labeling technique (**Fig. 6**). ABCB1 (multidrug resistance 1) was specifically photoaffinity-labeled with 8-azido-ATP as reported previously (lane 6) (28). ABCG1 was photoaffinity-labeled with 25 μM 8-azido-[α-$^{32}$P]ATP (lane 2), and the labeling was inhibited by ATP or ADP (lanes 3 and 4), demonstrating that ABCG1 can bind both ATP and ADP. The ABCG1-K120M mutant was not photoaffinity-labeled with 25 μM 8-azido-[α-$^{32}$P]ATP (lane 5), indicating that WalkerA lysine affects the nucleotide binding of ABCG1.

### Efflux of cholesterol and phospholipids by ABCG1

Because *ABCG1* mRNA expression is induced by ligands for RXR/LXR as is *ABCA1* mRNA expression (7, 14, 15), ABCG1 may be involved in cholesterol and phospholipid homeostasis in the cell. To examine the possibility that ABCG1 is involved in lipid efflux from cells as is ABCA1,

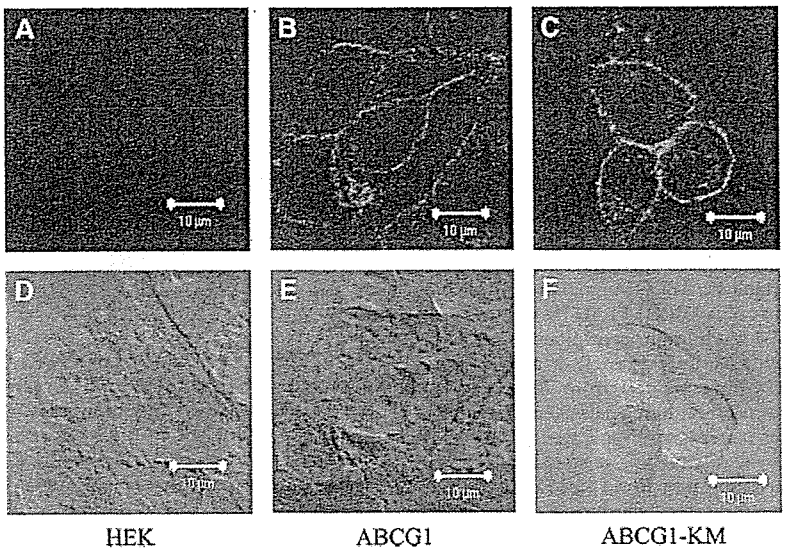


**Fig. 2.** Subcellular localization of ABCG1. HEK293 cells (A, D), HEK/ABCG1 cells (B, E), and HEK/ABCG1-K120M cells (C, F) were permeabilized with Triton X-100 and reacted with rabbit polyclonal anti-ABCG1 antibody and Alexa 488-conjugated anti-rabbit IgG. Immunostained images are shown in A–C, and differential interference contrast images are shown in D–F.

cholesterol pools of recycling endosomes were labeled with [$^3$H]cholesterol by incubating cells in DMEM containing 10% FBS and [$^3$H]cholesterol and the fractional release of [$^3$H]cholesterol to the medium after 4 h of incubation was measured (**Fig. 7A**). Host HEK293 cells did not mediate the efflux of [$^3$H]cholesterol in the absence (1.3 ± 0.41%) or presence (1.4 ± 0.41%) of apoA-I. HDL enhanced the efflux of [$^3$H]cholesterol from cells (5.6 ± 0.56%). A cell line (HEK/ABCA1) stably expressing ABCA1 mediated cholesterol efflux in the presence of apoA-I (7.3 ± 1.7%) but not in the absence of apoA-I (2.2 ± 0.45%). HEK/ABCA1 mediated slightly the higher efflux of cholesterol (7.8 ± 1.5%) than host HEK293 cells in the presence of HDL, but the difference was not significant. As reported previously (13, 19), the presence of HDL enhanced the cholesterol efflux from HEK/ABCG1 cells (11 ± 1.0%) compared with host HEK293 and HEK/ABCA1 cells. Noteworthy, HEK/ABCG1 mediated significantly higher efflux of cholesterol compared with the host HEK293 cells in the absence (3.6 ± 0.78%) or presence (4.3 ± 0.22%) of apoA-I. The release of [$^3$H]cholesterol in the absence (2.6 ± 0.74%) or presence of apoA-I (2.1 ± 0.84%) or HDL (6.8 ± 0.84%) from HEK/ABCG1-K120M cells did not significantly differ from that from host HEK293 cells. Furthermore, cells were labeled with [$^3$H]choline, and the fractional release of [$^3$H]choline phospholipids to the medium after 4 h of incubation was measured (Fig. 7B). Host HEK293 cells did not mediate the efflux of [$^3$H]choline phospholipids in the absence (0.30 ± 0.079%) or presence of apoA-I (0.23 ± 0.050%) or HDL (0.25 ± 0.080%). HEK/ABCA1 mediated phospholipid efflux in the presence of apoA-I (0.73 ± 0.15%) or HDL (0.78 ± 0.12%) but not in the absence of apoA-I (0.25 ± 0.036%). HEK/ABCG1 cells mediated higher efflux of

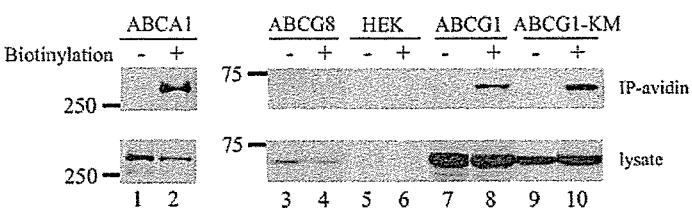


**Fig. 3.** Biotinylation of ABCG1. HEK/ABCA1 cells (lanes 1, 2), HEK/ABCG8 cells (lanes 3, 4), HEK293 cells (lanes 5, 6), HEK/ABCG1 cells (lanes 7, 8), and HEK/ABCG1-K120M cells (lanes 9, 10) were treated without (lanes 1, 3, 5, 7, 9) or with sulfo-*N*-hydroxysuccinimidobiotin (lanes 2, 4, 6, 8, and 10), and cell lysates were prepared. Biotinylated surface proteins were precipitated with avidin agarose from 100 μg of cell lysates. Cell lysates (20 μg of protein; lower panel) and precipitated surface proteins (upper panel) were separated by 10% polyacrylamide gel electrophoresis and detected with mouse monoclonal anti-ABCA1 antibody (lanes 1, 2), rabbit polyclonal anti-ABCG8 antibody (lanes 3, 4), or rabbit polyclonal anti-ABCG1 antibody (lanes 5–10). IP, immunoprecipitate.





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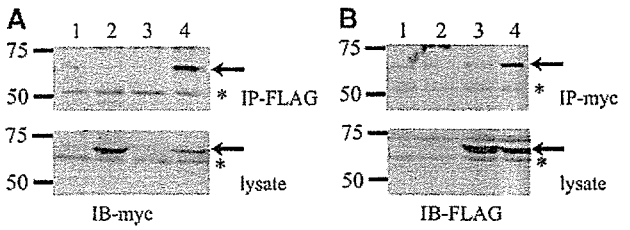


**Fig. 4.** Homomultimerization of ABCG1. HEK293 cells were transfected with mock plasmid (lane 1), ABCG1-myc (lane 2), ABCG1-FLAG (lane 3), or ABCG1-myc plus ABCG1-FLAG (lane 4), and cell lysates were prepared. ABCG1 was immunoprecipitated with rabbit polyclonal anti-FLAG antibody (A, upper panel) or mouse monoclonal anti-myc antibody (B, upper panel) from 700 μg of cell lysates. Cell lysates (20 μg of protein; lower panels) and immunoprecipitated proteins (upper panels) were separated by 10% polyacrylamide gel electrophoresis. ABCG1 was detected with mouse monoclonal anti-myc (A) or rabbit polyclonal anti-FLAG (B) antibody. Nonspecific bands are indicated by asterisks. IB, immunoblot; IP, immunoprecipitate.

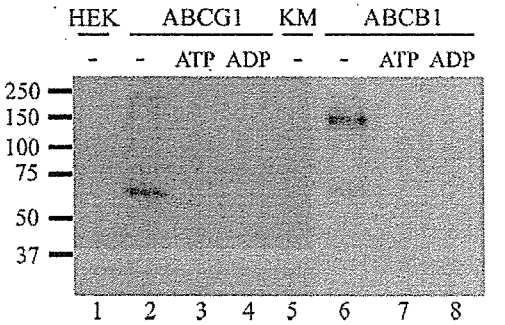


**Fig. 6.** 8-Azido-ATP binding of ABCG1. Membrane proteins (20 μg of proteins) from HEK293 cells (lane 1), HEK/ABCG1 cells (lanes 2–4), HEK/ABCG1-K120M cells (lane 5), and HEK293 cells transiently expressing ABCB1 (multidrug resistance 1) (lanes 6–8) were incubated with 25 μM 8-azido-[α-$^{32}$P]ATP in the absence (lanes 1, 2, 5, 6) or presence of 5 mM ATP (lanes 3, 7) or ADP (lanes 4, 8) for 10 min on ice followed by ultraviolet light irradiation. ABCG1 and histidine-tagged ABCB1 were immunoprecipitated with goat polyclonal anti-ABCG1 (lanes 1–5) and mouse monoclonal anti-PentaHis (lanes 6–8) antibody, respectively. Samples were separated by 10% polyacrylamide gel electrophoresis and autoradiographed.

phospholipids compared with the host HEK293 cells in the absence (0.55 ± 0.14%) or presence of apoA-I (0.58 ± 0.010%) or HDL (0.54 ± 0.014%). The release of [$^3$H]choline phospholipids in the absence (0.18 ± 0.017%) or presence of apoA-I (0.21 ± 0.023%) or HDL (0.23 ± 0.015%) from HEK/ABCG1-K120M cells did not significantly differ from that from host HEK293 cells. These results suggest that intact NBF is essential for the efflux of cholesterol and phospholipids.

Because labeling of intracellular cholesterol pools varies with methods used to deliver the labeled cholesterol or its precursors (29, 30), we further examined the efflux of cellular total cholesterol and choline phospholipids using colorimetric enzyme assays (31) (**Fig. 8**), by which we successfully measured apoA-I-dependent lipid efflux by ABCA1 and ABCA7 (32–34). The medium of host HEK293 cells contained minimal amounts of cholesterol and phospholipids. As reported previously (20), HEK/ABCA1 exhibited cholesterol and choline phospholipid efflux in an apoA-I-dependent manner. The medium of HEK/ABCG1 contained cholesterol (1.2 ± 0.025 μg/well) and choline phospholipid (0.98 ± 0.18 μg/well) at levels as high as that of HEK/ABCA1 in the presence of apoA-I (1.0 ± 0.072 and 0.84 ± 0.21 μg/well). However, surprisingly, the medium of HEK/ABCG1 contained cholesterol (0.93 ± 0.21 μg/well) and choline phospholipids (0.96 ± 0.28 μg/well) even in the absence of apoA-I. The lipid efflux by ABCA1 and ABCG1 increased in a time-dependent manner over 48 h (see supplementary Fig. I). The lipid contents were markedly reduced in the medium of HEK/ABCG1-K120M, suggesting that the binding and/or hydrolysis of ATP is required for the efflux of cholesterol and choline phospholipids by ABCG1. The intracellular contents of free cholesterol, total cholesterol, and phospholipids of HEK/ABCG1 were 15 ± 0.73, 16 ± 0.58, and 61 ± 0.50 μg/well, respectively, and not significantly different from those of HEK293 host cells. Therefore, 5.5% and 1.5% of cellular total cholesterol and phospholipids were

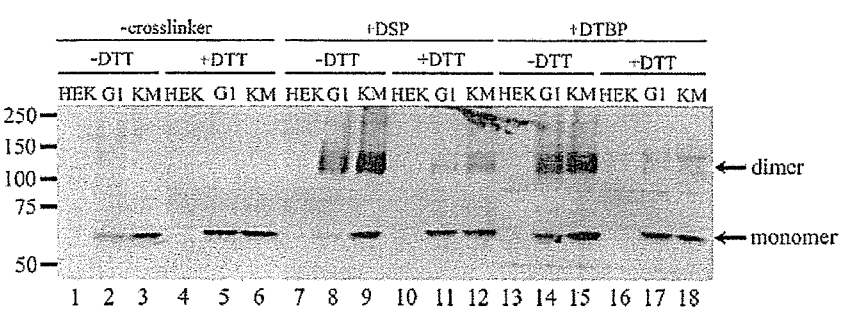


**Fig. 5.** Homodimerization of ABCG1. HEK293 cells (lanes 1, 4, 7, 10, 13, 16), HEK/ABCG1 cells (lanes 2, 5, 8, 11, 14, 17), and HEK/ABCG1-K120M cells (lanes 3, 6, 9, 12, 15, 18) were treated without (lanes 1–6) or with dithiobis (succinimidylpropionate) (DSP; lanes 7–12) or dimethyl 3,3′-dithiobispropionimidate-HCl (DTBP; lanes 13–18). Cell lysates (20 μg of protein) were treated without (lanes 1–3, 7–9, 13–15) or with DTT (lanes 4–6, 10–12, 16–18). Samples were separated by 7% polyacrylamide gel electrophoresis and detected with rabbit polyclonal anti-ABCG1 antibody.

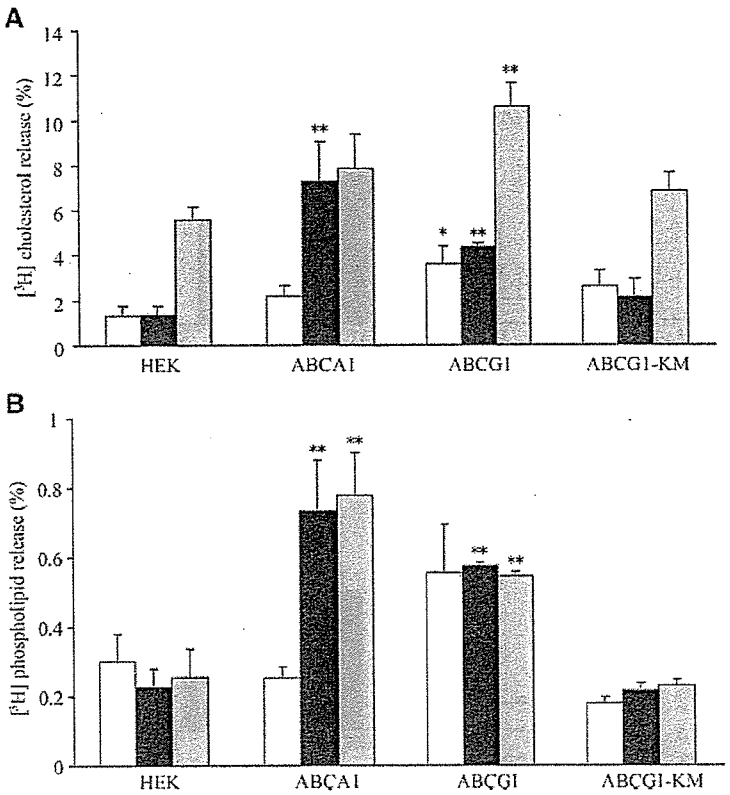


Fig. 7. Efflux of fractional [$^3$H]cholesterol or [$^3$H]-choline phospholipids by ABCG1. Cells were labeled with [$^3$H]cholesterol or [$^3$H]choline in DMEM containing 10% FBS for 24 h, and the efflux of [$^3$H]cholesterol (A) or [$^3$H]choline phospholipids (B) from HEK293 cells, HEK/ABCA1 cells, HEK/ABCG1 cells, or HEK/ABCG1-K120M cells during 4 h in the presence of 0.02% BSA alone (white bars), 0.02% BSA plus 10 μg/ml apolipoprotein A-I (apoA-I; black bars), or 0.02% BSA plus 20 μg/ml HDL (gray bars) was analyzed. Total [$^3$H]-choline phospholipids (cpm) in the medium were 196 ± 16 (BSA), 191 ± 50 (BSA + apoA-I), and 228 ± 77 (BSA + HDL) in HEK293 cells; 244 ± 15 (BSA), 785 ± 112 (BSA + apoA-I), and 752 ± 63 (BSA + HDL) in HEK/ABCA1 cells; 456 ± 44 (BSA), 504 ± 37 (BSA + apoA-I), and 554 ± 36 (BSA + HDL) in HEK/ABCG1 cells; and 122 ± 29 (BSA), 120 ± 14 (BSA + apoA-I), and 165 ± 8 (BSA + HDL) in HEK/ABCG1-K120M cells. Experiments were performed in triplicate, and average values are represented (±SD) as the percentage of the radioactivity in medium relative to the total radioactivity in cells and medium. * $P < 0.05$, ** $P < 0.01$ compared with host HEK293 cells.

secreted from HEK/ABCG1 in 48 h. In these experiments, expression levels of the wild-type and mutant ABCG1 were similar, and addition of apoA-I did not affect the expression (data not shown). Cell viabilities of HEK293 and HEK/ABCG1 cells [examined by 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt assay] were similar at 48 h and >98% (data not shown). To confirm that these results did not reflect a clonal bias, we examined another stable cell line of HEK/ABCG1 and found that they mediated the efflux of cholesterol and phospholipids in a similar manner (see supplementary Fig. II).

### BSA-dependent efflux of lipids by ABCG1

ABCG1 mediated the efflux of cholesterol and phospholipids even without apoA-I being added to the medium. Because the medium was supplemented with 0.02% BSA and BSA is believed to serve as a cholesterol acceptor in sperm capacitation (35), we examined the possibility that BSA works as an acceptor of cholesterol and phospholipids transported by ABCG1 (**Fig. 9A, B**). The cholesterol and phospholipid efflux by ABCG1 increased in a BSA concentration-dependent manner up to 0.02%. Surprisingly, a substantial efflux of cholesterol (0.61 ± 0.17 μg/well) and phospholipids (0.40 ± 0.023 μg/well) was observed even without BSA being added to the medium. Next, we examined whether serum proteins, including albumin, still remained in the medium after cells were washed with fresh medium: we found that significant amounts (~0.0002%) of serum albumin from FBS remained, as judged from Western blotting (see supplementary Fig. III). This suggests that serum albumin may function as an acceptor for cholesterol and phospholipids transported by ABCG1.

### Efflux of SM by ABCG1

HEK/ABCA1 and HEK/ABCG1 mediated the efflux of similar amounts of lipids with similar ratios between cholesterol and phospholipids in the presence of apoA-I, as shown in Fig. 8. The difference between the functions of ABCA1 and ABCG1 was that ABCG1 mediated the efflux even in the absence of apoA-I. We speculated that the mechanism of lipid efflux mediated by ABCG1 was different from that by ABCA1 and that phospholipid species secreted by ABCA1 and ABCG1 might be different. It has been reported that ABCA1 mediates the efflux of phospholipid, mostly PC (36). We compared the species of choline phospholipids transported by ABCA1 in the presence of apoA-I and by ABCG1 in the presence of BSA by analyzing molecular species of phospholipids in the medium with mass spectrometry (**Fig. 10**).

The media of HEK/ABCG1 cells in the presence of BSA (Fig. 10B) and HEK/ABCA1 cells in the presence of apoA-I (Fig. 10C) contained higher amounts of various choline phospholipids (SM 16:0-18:1, PC 16:0-16:1, PC 16:0-18:1, PC 18:0-18:2, and SM 24:1-18:1) than that of the host HEK293 cells in the presence of BSA (Fig. 10A). The major differences between HEK/ABCG1 and HEK/ABCA1 are

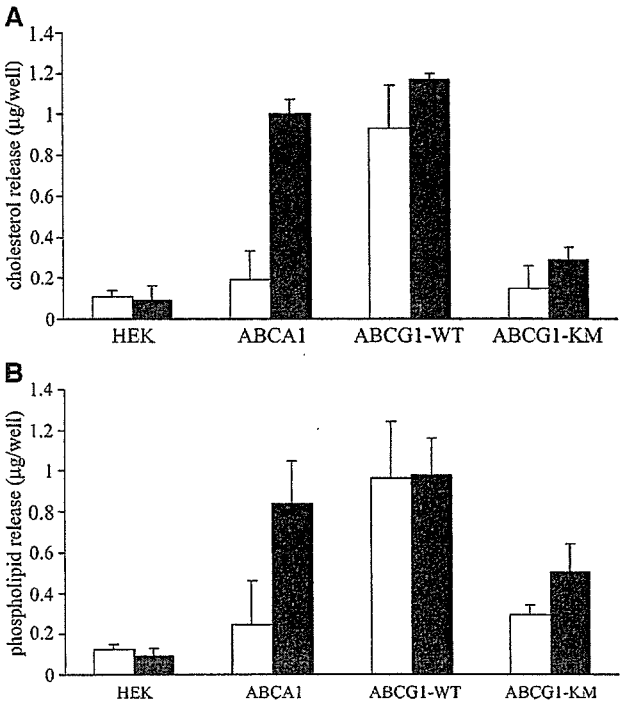


**Fig. 8.** Efflux of cellular cholesterol and phospholipids by ABCG1. The efflux of cholesterol (A) and phospholipids (B) from HEK293 cells, HEK/ABCA1 cells, HEK/ABCG1 cells, and HEK/ABCG1-K120M cells during 48 h in the presence of 0.02% BSA alone (white bars) or 0.02% BSA plus 10 μg/ml apoA-I (black bars) was analyzed. Experiments were performed in triplicate, and average values are represented (±SD).

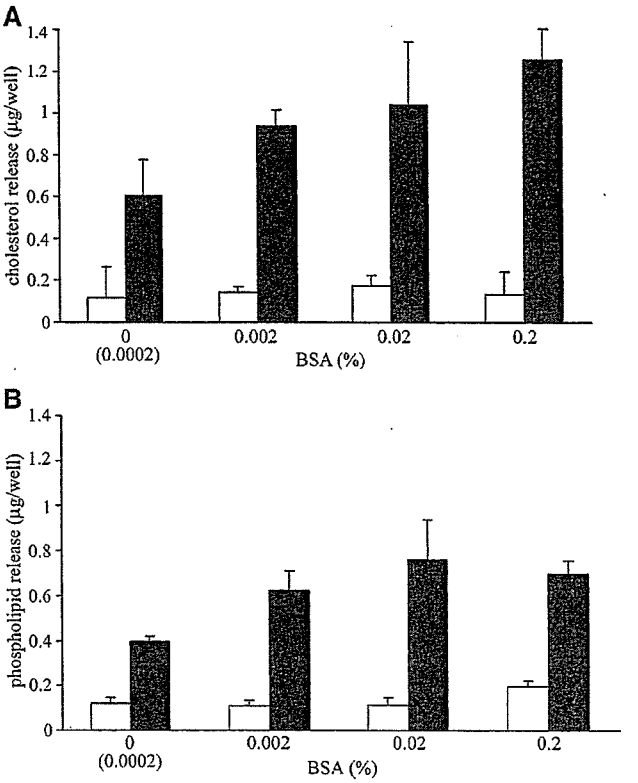


**Fig. 9.** Dependence on BSA concentration of the efflux of cholesterol and phospholipids by ABCG1. The efflux of cholesterol (A) and phospholipids (B) from HEK293 cells (white bars) or HEK/ABCG1 cells (black bars) during 24 h with the indicated concentrations of BSA was analyzed. Even in the absence of added BSA, ~0.0002% BSA was found, as judged from Western blotting against BSA, because BSA from FBS remained. Experiments were performed in triplicate, and average values are represented (±SD).

the peak heights of SM 16:0-18:1 and SM 24:1-18:1. In the medium of HEK/ABCG1 cells, the peak height of SM 16:0-18:1 was much higher than that of PC 16:0-16:1, whereas it was lower than that of PC 16:0-16:1 in the medium of HEK/ABCA1 cells. The peak height of SM 24:1-18:1 was also higher than that of PC 18:0-18:2 in the medium of HEK/ABCG1 cells, whereas it was lower than that of PC 18:0-18:2 in the medium of HEK/ABCA1 cells. Although ion peaks from a triple quadupole mass spectrometer do not allow for direct comparison between phospholipid species, the relationship between the amount of bovine brain SM and the peak height of SM 16:0-18:1 was linear from 25 to 900 μg/ml, as shown in Fig. 10C (inset). Furthermore, the relationship of the peak height of SM 16:0 was linear from 1 to 100 pmol/μl compared with 1 pmol/μl PC 16:0-16:0 (see supplementary Fig. IV). Therefore, a higher content of SM 16:0-18:1 and SM 24:1-18:1 in the medium of HEK/ABCG1 cells than in the medium of HEK/ABCA1 cells would be dependable. The medium of HEK/ABCG1 cells also included significant amounts of lysoPC 14:0, lysoPC 16:0, and lysoPC 18:1 compared with that of host HEK293 cells (data not shown), but it is not clear whether these lysoPCs were secreted from cells or converted from PC in the medium. These results suggest that ABCG1 preferentially secretes SM compared with PC, whereas ABCA1 preferentially secretes PC compared with SM.

We further examined whether ABCG1 mediated the efflux of SM (**Fig. 11**). The relative radioactivity of SM against PC secreted from HEK/ABCG1 cells in the presence of BSA (1.3 ± 0.42), apoA-I (1.1 ± 0.30), or HDL (1.3 ± 0.30) was significantly higher than that from HEK/ABCA1 in the presence of apoA-I (0.14 ± 0.045) or HDL (0.16 ± 0.012). This shows that ABCG1 mediates the efflux of SM preferentially, whereas ABCA1 mediates the efflux of PC preferentially.

## DISCUSSION

In this study, we established HEK293 cells stably expressing human ABCG1 and showed that ABCG1 localizes to the plasma membrane and mediates the efflux of cholesterol and phospholipids (preferentially SM). ABCG1 has been reported to be expressed as several splicing variants in the N-terminal region and within NBF. *ABCG1* cDNA, cloned from a fetal liver cDNA library in this study, has the same N-terminal sequence as that originally reported by Chen et al. (6). This type of variant was identified in

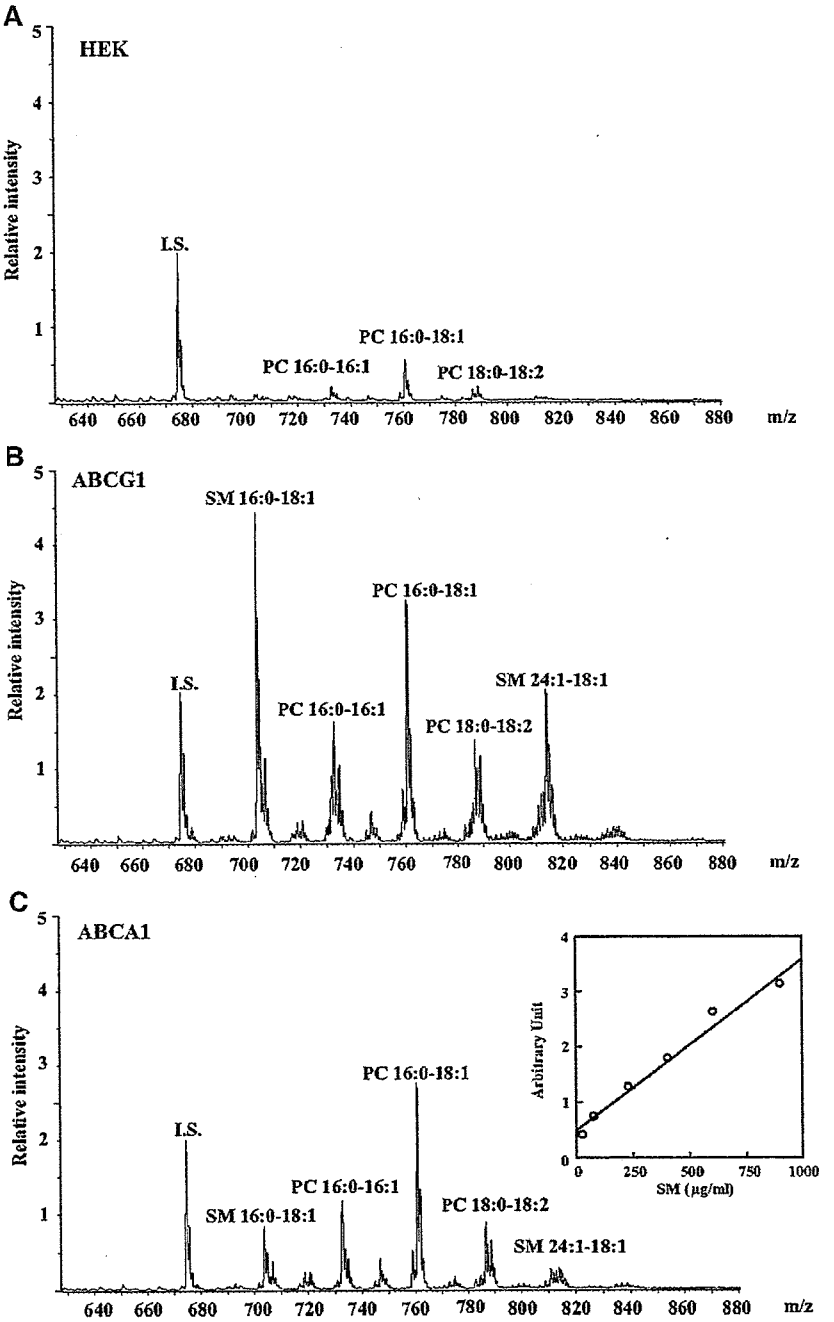


**Fig. 10.** Positive-ion electrospray ionization MS spectra of choline phospholipid molecular species in lipid extracts of the culture media. Phospholipids were extracted from the medium of HEK293 cells (A), HEK/ABCG1 cells incubated in the presence of 0.02% BSA (B), or HEK/ABCA1 cells incubated in the presence of 0.02% BSA plus 10 μg/ml apoA-I for 48 h (C). Aliquots of chloroform extracts were infused directly into the electrospray ionization ion source using an UltiMate HPLC system at a flow rate of 4 μl/min. Positive-ion electrospray ionization of lipid extracts of the cell medium was performed as described in Materials and Methods. Individual molecular species were identified using tandem mass spectrometry. The internal standard (I.S.) is 14:1-14:1 phosphatidylcholine (PC) (*m/z* 674.5) and is presented as a relative intensity value of 2. The inset in C shows the relationship between peak height and amount of sphingomyelin (SM).

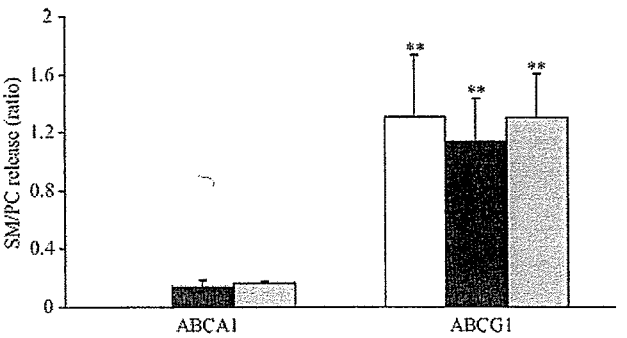


**Fig. 11.** Relative radioactivity of SM against PC. Cells were labeled with [$^3$H]choline in DMEM containing 10% FBS for 24 h, and the [$^3$H]choline phospholipids were extracted by chloroform-methanol from the medium of HEK/ABCA1 or HEK/ABCG1 cells during 4 h in the presence of 0.02% BSA alone (white bars), 0.02% BSA plus 10 μg/ml apoA-I (black bars), or 0.02% BSA plus 20 μg/ml HDL (gray bars). The extracted [$^3$H]choline phospholipids were separated by TLC, and the radioactivities of spots of PC and SM were counted. Experiments were performed in triplicate, and the relative radioactivities of SM/PC are represented (±SD). ** $P < 0.01$ compared with HEK/ABCA1 cells in the presence of apoA-I.

placenta and a macrophage cDNA library (12). The exogenously expressed ABCG1 in HEK293 cells showed a similar mobility on SDS-PAGE to the endogenous ABCG1 in THP-1 cells (Fig. 1), suggesting that the variant used in this study could be a major form in macrophages.

*N*-linked oligosaccharides are involved in the quality control of proteins by the calnexin and/or calreticulin cycle (37), and the expression of several ABC proteins has been reported to be regulated by the endoplasmic reticulum lectin (38). The isolated *ABCG1* cDNA does not contain any putative *N*-linked glycosylation sites, as reported previously in human (6) and mouse (25, 26) ABCG1, unlike that reported by Chen et al. (6). The experiment with *N*-glycosidases clearly showed that the endogenous ABCG1 in THP-1 cells is not modified by *N*-linked oligosaccharides (Fig. 1). Therefore, the major form of ABCG1 in vivo may not contain an *N*-linked glycosylation site and may function without *N*-linked oligosaccharides. It was reported that functional NBD is necessary for the trafficking of ABCG1 to the plasma membrane, because the G121A mutation within the WalkerA motif of ABCG1 abolished the cell surface expression (13). However, ABCG1-K120M localized to the plasma membrane (Fig. 2), suggesting that ATP binding and/or hydrolysis is not essential for the surface expression.

Recently, it was suggested that ABCG2 forms a multimer (27). However, cross-linking experiments show that most of the ABCG1 works as a homodimer of 60–65 kDa in an unglycosylated form, unlike ABCG2. Western blot analysis detected several bands (120–150 kDa) after cross-linking (Fig. 5). Vaughan and Oram (13) also detected similar bands by cross-linking experiment with disuccinimidyl suberate. Because we could not detect any endogenous ABCG subfamily protein (ABCG1, ABCG2, ABCG4, ABCG5, or ABCG8) in HEK293 cells by Western blot (data not shown), it is unlikely that ABCG1 forms heterodimers with endogenous ABCG subfamily proteins. ABCG1 may interact with other endogenous proteins. It was reported that ABCG1 is expressed as a protein of ~95 kDa in perinuclear structures within cholesterol-laden macrophages and is present in foamy macrophages within atherosclerotic plaques (17). It was also reported that ABCG1 is expressed on the cell surface and in intracellular compartments of cholesterol-laden macrophages as a 110 kDa protein (14). It is not clear whether the type of splicing variant and/or *N*-glycosyl modification accounts for the differences in the subcellular localization and molecular size of ABCG1 expressed in cholesterol-laden macrophages.

HEK/ABCG1 cells secrete cholesterol and choline phospholipids as efficiently as HEK/ABCA1 cells in the presence of apoA-I (Fig. 8). Because the lipid efflux by ABCG1 is impaired by the amino acid substitution of lysine in NBF, as reported for ABCA1 (39), the efflux is dependent on the function of ABCG1 operated by ATP binding and/or hydrolysis. Notably, ABCG1 mediates cholesterol and phospholipid efflux even in the absence of apoA-I, whereas lipid efflux by ABCA1 is dependent on the presence of apoA-I. Lipid efflux mediated by ABCG1 was apparently dependent on the presence of BSA (Fig. 9), suggesting that serum albumin can function as an acceptor for cholesterol and phospholipids transported by ABCG1. In the presence of 0.002% BSA, ABCG1 mediated the efflux of 0.94 μg/ml cholesterol, which suggests that the molar ratio of cholesterol and BSA is 7:1 in the medium. Phospholipids in the medium may enhance the binding of cholesterol to BSA. Alternatively, some proteins secreted from HEK293 cells may function as acceptors as well. However, even if that is the case, these molecules cannot serve as an acceptor for lipids secreted by ABCA1, suggesting that ABCG1 has broader acceptor specificity than ABCA1 has.

Recently, Cignarella et al. (40) reported cholesterol efflux from monocyte-derived macrophages in the absence of acceptors correlated with the induction of ABCG1 expression. It was also reported that ABCG1 mediates the efflux of cholesterol and phospholipids from cells to HDL-2 or HDL-3 (19). Because the expression level of ABCG1 in THP-1 cells induced by the LXR ligand was comparable to that in HEK/ABCG1 cells, direct cholesterol efflux by ABCG1 from macrophages could be important within atherosclerotic lesions, in which the amounts of lipid acceptors are limited. Furthermore, in the presence of proper acceptors of secreted cholesterol in plasma, such as HDL-2 and HDL-3, direct efflux by ABCG1 would play an important function in cholesterol and phospholipid secretion.

The mass analysis revealed that ABCG1 secretes SM as well as PC into medium from HEK/ABCG1 cells. The main constituent of the plasma membrane is PC, and it was reported that, when incubated with fibroblasts, lipid-free apoA-I produces lipoproteins containing 69% PC and 18% SM (36). Rough total peak heights of PCs in Fig. 10C can be calculated to be several fold that of

SMs. The major differences between phospholipids of HEK/ABCG1 and HEK/ABCA1 cells are the peak heights of SM 16:0-18:1 and SM 24:1-18:1. Although ion peaks from a triple quadrupole mass spectrometer do not always allow for quantitation and ion intensities of the signals are to be corrected (41), there was a linear relationship between the amount of SM and the peak height. Furthermore, the ratio of SM to PC effluxed by ABCG1 was eight times higher than that effluxed by ABCA1 (Fig. 11). From these results, it is assumed that ABCG1 preferentially secretes SM into the medium from HEK/ABCG1 cells. SM is synthesized in the lumen of the Golgi apparatus (42) and moved to the outer leaflet of the plasma membrane by vesicular membrane transport (43). SM has high affinity for cholesterol and tends to form a complex with cholesterol in the outer leaflet of the plasma membrane. ABCG1 may secrete cholesterol together with SM into the medium from the outer leaflet of the plasma membrane. It is known that the LolCDE system catalyzes the release of lipoproteins anchored to the outer leaflet of the inner membrane through the N-terminal fatty acyl chains in *Escherichia coli* (44).

In this study, we showed that the amounts of cholesterol and phospholipids in the medium of HEK/ABCG1 cells after 48 h of incubation in the presence or absence of apoA-I are similar to those of HEK/ABCA1 cells in the presence of apoA-I, as shown in Fig. 8, whereas the efficiency of cholesterol and phospholipid efflux at 4 h was not so high, as shown in Fig. 7. In a previous study, cells expressing ABCG1 showed an efflux of cholesterol to HDL-2 and HDL-3 but not to lipid-poor apoA-I (13, 19). The cause of this discrepancy is not clear, but one possible explanation is that the amounts of free cholesterol and choline phospholipids in the medium were measured by colorimetric enzyme assays in Fig. 8, whereas cells were labeled with [$^3$H]cholesterol or [$^3$H]choline and radioactivity was measured in Fig. 7 and in previous studies. Because it was reported that the labeling of intracellular cholesterol pools varies with the methods used to deliver the labeled cholesterol or its precursors (29), ABCG1 and ABCA1 might mediate the efflux of cholesterol from different intracellular pools. Another possibility may be that cells stably expressing ABCG1, at levels comparable to cells derived from human macrophages, were used in this study, whereas cells transiently expressing ABCG1 were used in the previous study. Although further studies are necessary to understand the precise mechanism of lipid efflux by ABCG1, to our knowledge, this is the first report of a protein that mediates SM efflux.

In summary, we have demonstrated that ABCG1 localizes in the plasma membrane and mediates the efflux of cholesterol and phospholipids, especially SM. ABCA1 and ABCG1 expression is coordinately induced in macrophages in the LXR pathway, and ABCA1 mediates the efflux of cholesterol and phospholipids to apolipoproteins to form preβ-HDL. In macrophages, ABCG1 may be involved in the removal of excess lipids by mediating the efflux of cholesterol and phospholipids, especially SM.

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# 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 was impaired, whereas wild-type and N568D, G1221S, and L1580P mutant ABCA3 proteins trafficked to the LAMP3-positive intracellular vesicle, accompanied by 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 dipalmitoylphosphatidylcholine, 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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[2] 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.

factant deficiency lie in various locations on the *ABCA3* gene (Fig. 1*A*).

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, W1142*X*, and Ins1518fs (abbreviation of Ins1518fs/ter1519 in this study), see Fig. 1*A*), 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 (25) 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% $CO_2$ in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum and penicillin/streptomycin. HEK293 cells ($3 \times 10^5$) were seeded into 35-mm dishes with poly-L-lysine-coated cover glass. 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 stably expressing ABCA3-GFP were fixed, permeabilized with 0.5% Nonidet P-40 in phosphate-buffered saline, blocked in 5% bovine serum albumin in phosphate-buffered saline, and labeled with mouse anti-human LAMP3 antibody (Chemicon) and Cy3-conjugated sheep anti-mouse IgG antibody (Amersham Biosciences). For transfection into MLE12 cells, cells were grown in Dulbecco's modified Eagle's medium/F12 medium (Invitrogen) supplemented with 2% fetal calf serum and penicillin/streptomycin and transfected using Lipofectamine 2000 reagent (Invitrogen).

*Glycosylation of ABCA3-GFP Protein and Mutants*—HEK293 cells ($3 \times 10^6$) were seeded into 100-mm dishes 24 h before transfection. Forty eight h after transfection with pEGFP vectors (6.25 μg) using FuGENE reagent, cells were homogenized in 50 mM Tris-HCl (pH 7.5) buffer containing complete protease inhibitor mixture (Roche Applied Science), by a Potter-Elvehjem homogenizer, and then centrifuged at 1,000 × *g* for 10 min to obtain post-nuclear supernatant (PNS). PNS was further centrifuged at 100,000 × *g* for 1 h to obtain the total membrane fraction. Ten μg of total membrane fraction was treated with 1 unit of peptide *N*-glycosidase F (PNGase F) or 5 milliunits of endoglycosidase H (Endo H) for 30 min at 37 °C. The deglycosylated proteins were separated by SDS-PAGE (5%) and analyzed by immunoblot analysis by using anti-GFP monoclonal antibody (Santa Cruz Biotechnology). The amount of each protein was quantified by measuring the density of the band using NIH image software, and 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; see Fig. 3*C*) was represented as a percentage of total band I plus band II (Endo H-sensitive 210-kDa protein except for 180-kDa Ins1518fs protein). Data are shown as means ± S.D. ($n = 3$). Statistical analysis was performed by the Bonferroni/Dunn procedure for post hoc testing.

*Establishment of HEK293 Cells and A549 Cells Stably Expressing ABCA3-GFP and Mutants*—HEK293 cells ($3 \times 10^5$) were seeded into 35-mm dishes and after 24 h were transfected with linearized wild-type pCAGIpuro-ABCA3GFP or mutant plasmid (1 μg) by PvuI using FuGENE reagent. A549 cells were grown at 37 °C under 5% $CO_2$ in RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum and penicillin/streptomycin. A549 cells ($3 \times 10^6$) were seeded into 100-mm dishes. After 24 h, cells were transfected with 6.25 μg of plasmid. Forty eight h after transfection, cells were trypsinized and seeded into 100-mm dishes and selected by 2.5 μg/ml puromycin for 7–10 days. Single colonies were isolated, and the expression of ABCA3-GFP or mutants was examined by immunoblot analysis and confocal microscopy.

*Vanadate-induced Nucleotide Trapping of ABCA3-GFP and Mutants with 8-Azido-[α-$^{32}$P]ATP*—HEK293 cells stably 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 $N_2$ 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 20,000 × *g* membrane fraction. A 20,000 × *g* membrane fraction (20–30 μg of protein) was incubated with 10 μM 8-azido-[α-$^{32}$P]ATP, 2 mM ouabain, 0.1 mM EGTA, 3 mM $MgCl_2$, 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 $MgCl_2$. 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 10 μl of Tris-HCl buffer containing 0.1 mM EGTA and 1 mM $MgCl_2$ and irradiated for 10 min (at 254 nm, 8.2 milliwatts/cm$^2$) on ice. The samples were then electrophoresed on a 5% SDS-polyacryl-

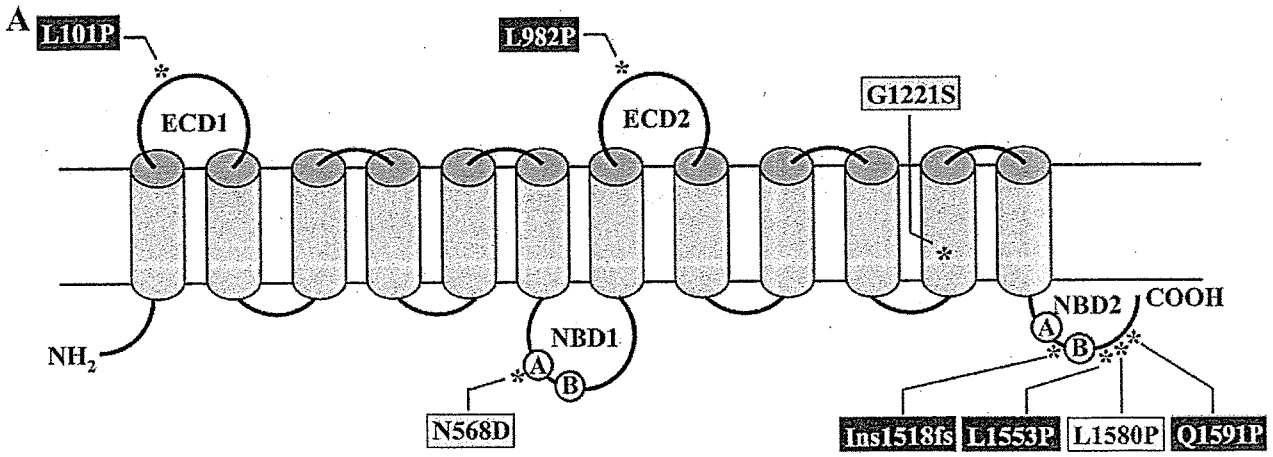
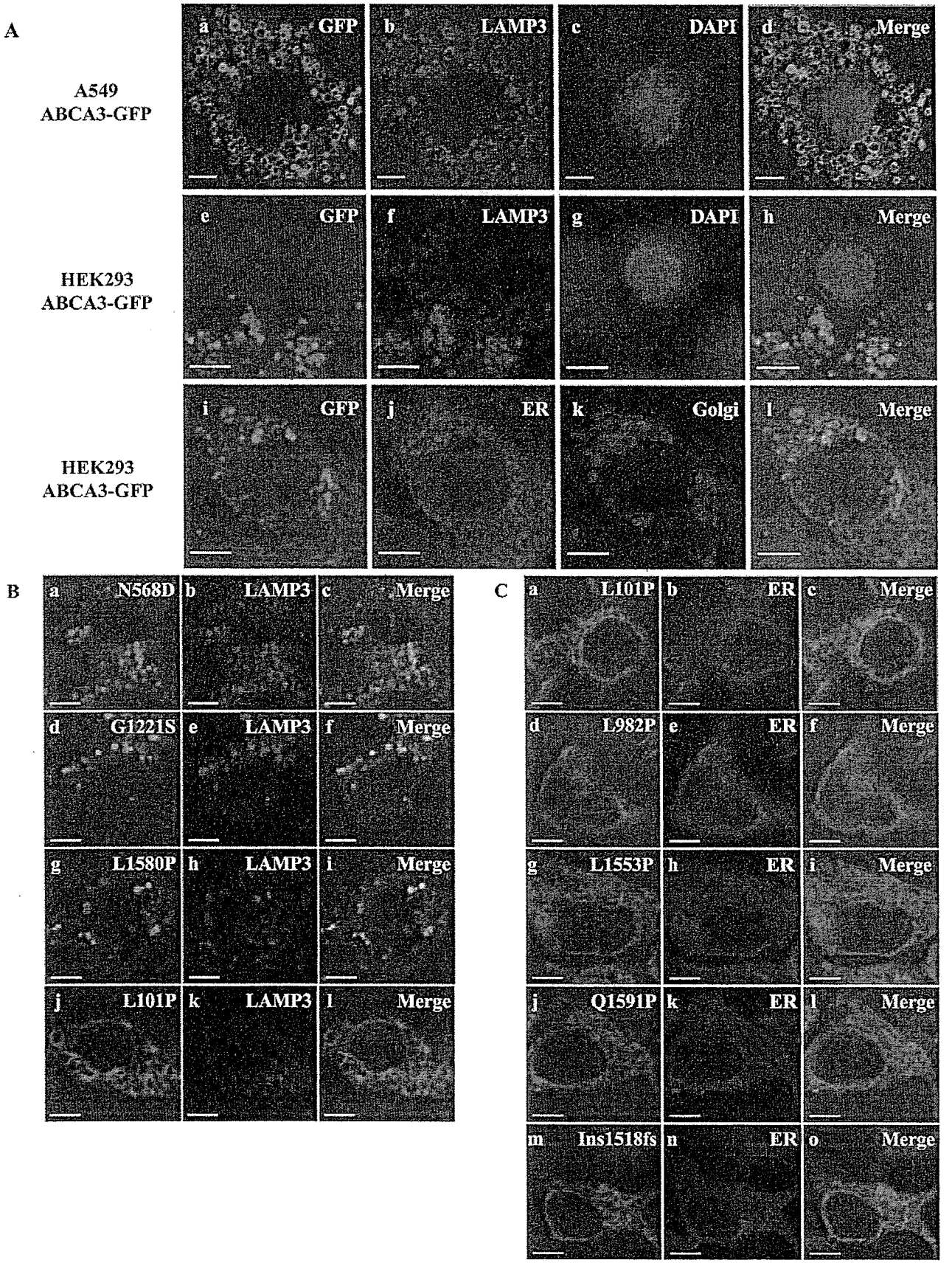



FIGURE 1. **Structural model of ABCA3 protein and alignment of partial amino acid sequence of ABCA subfamily.** *A*, the locations of fatal surfactant deficiency mutations characterized in this study are indicated. ABCA3 is constituted by 12 putative membrane-spanning helices, two extracellular domains (*ECD*), and two nucleotide binding domains (*NBD*). *Circled A* and *B* indicate Walker A and Walker B motifs, respectively, in the two nucleotide binding domains. *Closed boxes* and *gray boxes* indicate type I and type II mutations, respectively. The Ins1518fs mutant protein lacks the C-terminal polypeptide containing the Walker B sequence of NBD-2. C-terminal amino acid sequences of wild-type and Ins1518fs mutant protein are shown. *B–D*, alignments of amino acid sequence of Walker A motif (indicated by *line*) in NBD-1 (*B*), 11th transmembrane segment (TM-11) (*C*), and partial amino acid sequence of NBD-2 including Walker B motif (indicated by *line*) (*D*) of some members of the human ABCA subfamily are shown. The amino acids residues that are conserved in six transporters and in four or five transporters are shown by *asterisks* and *dots*, respectively.

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 ± 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-$^{32}$P]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-$^{32}$P]ATP, 2 mM ouabain, 0.1 mM EGTA, 3 mM $MgCl_2$, 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/cm$^2$) 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 ± S.D. ($n = 3$). Statistical analysis was performed as described above.



## 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-ABCA3GFP (expression plasmid for ABCA3 fused to GFP at its C terminus), most of the GFP fluorescence showed a ring-like appearance (Fig. 2*A*, *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. 2*A*, *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. 2*A*, *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. 2*A*, *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. 2*A*, *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. 1*A*). The N568D, G1221S, and L1580P mutant proteins were mainly localized to the LAMP3-positive intracellular vesicle membrane (Fig. 2*B*, *panels a–c*, *d–f*, and *g–i*), similar to wild-type ABCA3 protein (Fig. 2*A*, *panels e–h*). In contrast, the L101P, L982P, L1553P, Q1591P, and Ins1518fs mutant proteins were barely detectable at the LAMP3-positive intracellular vesicle membrane (Fig. 2*B panels j–l* for L101P and data not shown for others). The GFP fluorescence of these mutant proteins was merged with the DsRed2 fluorescence of the ER (Fig. 2*C*), suggesting that these mutant proteins are mainly localized at the ER. In another cell line, mouse lung epithelial MLE12 cells, transiently expressed GFP-tagged wild-type and N568D, G1221S, and L1580P mutant proteins were mainly localized at the intracellular vesicle membrane, 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. 3*A*) 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. 3*A*). 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. 3*A*). 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. 3*A*, 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 4552insT 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. 1*A*). 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. 3*A*).

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*-acetylglucosamine 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-

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 coverslips and processed for immunofluorescence labeling of LAMP3 (*panel b*), a marker of lamellar bodies and multivesicular bodies. Nuclei were counterstained by 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 by 4,6-diamidino-2-phenylindole (*g*). A merged image of *panels e–g* is shown in *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*. *B*, 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.

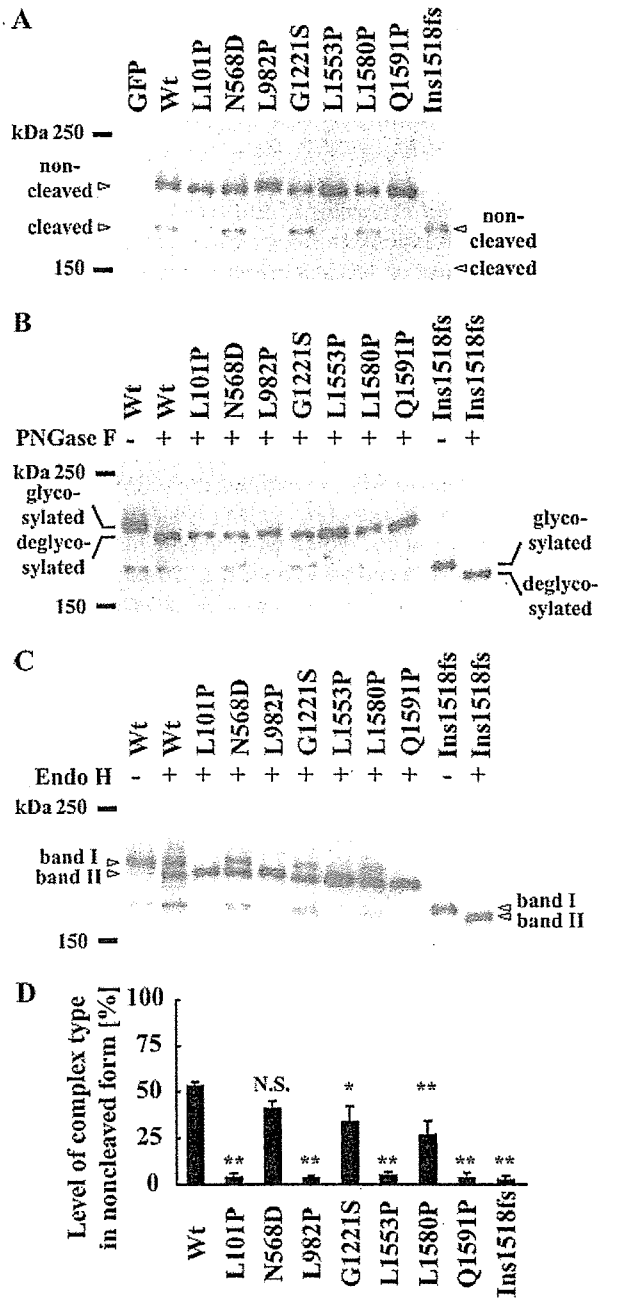



FIGURE 3. **Processing and glycosylation of wild-type ABCA3-GFP and mutant proteins in HEK293 cells.** *A*, 10 $\mu$g of total membrane fractions from HEK293 cells transiently transfected with GFP, wild-type (*Wt*) ABCA3-GFP, or mutants was subjected to SDS-PAGE (5%), transferred to PVDF membranes, and 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 $\mu$g 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 190 kDa in Ins1518fs) were deglycosylated by PNGase F and produced 210-kDa (wild-type and missense mutant) and 180-kDa (Ins1518fs) proteins. *C*, 10 $\mu$g of total membrane fraction with (+) or without (−) treatment of Endo H was subjected to SDS-PAGE and immunoblot analysis. *Band I* shows Endo H-insensitive 220-kDa ABCA3-GFP proteins (wild-type, N568D, L982P, and L1553P) containing complex-type sugar chains. ABCA3-GFP proteins modified with high mannose-type sugar chains were digested by Endo H and 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.

kDa wild-type ABCA3-GFP and the seven missense mutant (L101P, N568D, L982P, G1221S, L1553P, L1580P, and Q1591P) proteins to produce a 210-kDa deglycosylated protein (Fig. 3*B*). Electrophoretic mobility shift from 190 to 180 kDa also was observed in the Ins1518fs nonsense mutant protein (Fig. 3*B*), 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. 3*C*). 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-[$\alpha$-$^{32}$P]ATP is used as an ATP analog.

To examine vanadate-induced nucleotide trapping in ABCA3-GFP and the mutant proteins, HEK293 cells stably