Molecular Mechanisms of Subcellular Localization of ABCG5 and ABCG8

Takashi Hirata,1 Morio Okabe,1 Aya Kobayashi,1 Kazumitsu Ueda,1,2 and Michinori Matsuo1,1

1Laboratory of Cellular Biochemistry, Division of Applied Life Sciences, Kyoto University Graduate School of Agriculture, Kyoto 606-8502, Japan
2Institute for Integrated Cell-Material Sciences (iCeMS), Kyoto University, Kyoto 606-8502, Japan

Received October 1, 2008; Accepted October 30, 2008; Online Publication, March 7, 2009
[doi:10.1271/bbb.80694]

Human ABCG subfamily proteins ABCG1, ABCG2, ABCG4, ABCG5, and ABCG8 are half-type ATP-binding cassette (ABC) proteins that transport sterols or xenobiotics. ABCG1, ABCG2, and ABCG4 function as homodimers on the plasma membrane. In contrast, ABCG5 and ABCG8 function as heterodimers on the plasma membrane, and the homodimer of either ABCG5 or ABCG8 is retained in the endoplasmic reticulum (ER). To examine the molecular mechanisms of the regulated trafficking of ABCG5 and ABCG8, the subcellular localizations of chimeric proteins, fused with ABCG1 or ABCG2, were analyzed. Homodimers of chimeric proteins, in which the N-terminal cytosolic domain of ABCG1 or ABCG2 was fused to the C-terminal transmembrane domain of ABCG5 or ABCG8 localized to the plasma membrane, whereas chimeric proteins in which the N-terminal cytosolic domain of ABCG5 or ABCG8 was fused to the C-terminal transmembrane domain of ABCG1 or ABCG2 localized to the ER. Mutations in ER-retrieval motif-like sequences in ABCG5 or ABCG8 did not affect their subcellular localization. This suggests that the N-terminal cytosolic domains of ABCG5 and ABCG8 are involved in ER retention of their homodimers, and that novel ER-retention or -retrieval motifs exist within these domains.

Key words: cholesterol; plant sterol; ATP-binding cassette (ABC) protein; membrane trafficking; endoplasmic reticulum (ER) retention

ABCG subfamily proteins are half-type ATP-binding cassette (ABC) proteins, consisting of an N-terminal cytosolic nucleotide-binding domain (NBD) and a C-terminal transmembrane domain (TMD). There are five ABCG subfamily members in humans: ABCG1, ABCG2, ABCG4, ABCG5, and ABCG8. ABCG1 mediates the efflux of cholesterol, sphingomyelin, and phosphatidylcholine into high density lipoprotein (HDL) from cells.1,2) ABCG2 transports xenobiotics and confers on cancer cells multidrug-resistance against various anticancer drugs.3) ABCG4, which is induced by the nuclear receptors liver X receptor (LXR) and retinoid X receptor (RXR), like ABCG1, mediates the efflux of cholesterol into HDL.4) As reported previously, ABCG1 forms a homodimer and localizes to the plasma membrane.2) ABCG2 also forms a homodimer and localizes to the plasma membrane.4)5)

Cholesterol and noncholesterol sterols, including sitosterol, are taken every day in the diet. Whereas 50–60% of dietary cholesterol is absorbed from the intestine, less than 5% of noncholesterol sterols are absorbed. ABCG5 and ABCG8 are expressed in the apical membrane of the small intestine and liver,6)7) and mutations of ABCG5 or ABCG8 cause sitosterolemia, an autosomal recessive disorder characterized by the accumulation of both sterols in the plasma.7) Disruption of either ABCG5 or ABCG8 in mice exhibited the phenotype of sitosterolemia: the accumulation of plant sterols in the plasma, and reduced cholesterol excretion into the bile duct from the liver.5,8) On the other hand, overexpression of ABCG5 and ABCG8 decreased the fractional absorption and increased the biliary secretion of cholesterol.10,11) ABCG5 and ABCG8, expressed in cultured cells, mediated the efflux of sterols from the cells into bile salts added to the media.12) These findings suggest that ABCG5 and ABCG8 are involved in the control of the absorption of sterols from the intestine and the excretion of sterols from the liver.

It has been reported that ABCG5 and ABCG8 form a heterodimer localized to the plasma membrane, whereas the homodimers of ABCG5 and of ABCG8 are retained in the endoplasmic reticulum (ER).13,14) It is suggested that the heterodimer of ABCG5 and ABCG8 reaches the plasma membrane and mediates the efflux of cholesterol and sitosterol from cells, whereas their homodimers do not function in cholesterol efflux. Furthermore, nonsense mutations, which cause sitosterolemia, prevent heterodimer formation or trafficking to the plasma membrane.15) It is important to elucidate the regulation of the trafficking of ABCG5 and ABCG8 to understand sterol homeostasis in the body and the pathogenesis of sitosterolemia. It has been reported that the lectin-like chaperones calnexin and calreticulin interact with ABCG5 and ABCG8 and facilitate cell surface expression of the ABCG5/ABCG8 heterodimer.16,17) However, the molecular mechanism of ER retention of their homodimers is not yet clear. This study sought to elucidate the molecular mechanisms of ER retention of the ABCG5 and ABCG8 homodimers by analyzing the subcellular localization of chimeric ABCG subfamily proteins.
Materials and Methods

**Materials.** Rabbit polyclonal anti-ABCG1 antibody, mouse monoclonal anti-FLAG, and anti-myc antibodies were acquired from Santa Cruz Biotechnology (Santa Cruz, CA). Rat monoclonal anti-HA antibody was from Roche (Indianapolis, IN), and rabbit polyclonal anti-FLAG antibody was from Sigma (St. Louis, MO). The rabbit polyclonal anti-ABCG4 and anti-ABCG5 antibodies were raised against the cytoplasmic regions of human ABCG4 (amino acids 1–353) and ABCG5 (amino acids 1–389) respectively. Rabbit polyclonal anti-ABCG8 antibody was from Novas Biologicals (Littleton, CO). DSS (Dissuccinimidyl suberate) and BS3 (Bis[sulfosuccinimidyl]suberate) were from Thermo Fisher Scientific (Rockford, IL). Other chemicals were purchased from Sigma, GE Healthcare Bio-Sciences (Uppsala, Sweden), Wako Pure Chemical Industries (Osaka, Japan), and Nacalai Tesque (Kyoto, Japan). Human ABCG2 cDNA was a gift from Dr. Yoshikazu Sugimoto (Keio University).

**Cloning of human ABCG4, ABCG5, and ABCG8 cDNA.** ABCG4 cDNA was cloned from a human brain cDNA library (GenBank Accession no. NM_022169). The cDNAs of ABCG5 and ABCG8 were cloned from a human liver cDNA library (GenBank Accession nos. NM_022436 and NM_022437). The ABCG5, ABCG8, and N-terminal myc-tagged ABCG2 cDNA sequences were inserted into pcDNA3.1(+) (Invitrogen, Carlsbad, CA) to generate expression vectors pcDNA3.1/ABCG5, pcDNA3.1/ABCG8, and pcDNA3.1/myc-ABCG2 respectively. pcDNA3.1(+)A-HA was prepared by inserting the HA tag sequence instead of the myc- and His-tag sequences of pcDNA3.1(+)A-myc-His (Invitrogen). The stop codons of pcDNA3.1/ABCG4, pcDNA3.1/ABCG5, and pcDNA3.1/ABCG8 were mutated, and the cDNAs were inserted into pcDNA3.1(+)A-myc-His, pcDNA3.1(+)A-FLAG, or pcDNA3.1(+)A-HA for the expression of ABCG4, ABCG5, or ABCG8 fused with tags at their C-terminus.

**Cell culture.** HEK293 cells were grown in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) in 5% CO2 at 37°C.

**Transfection.** HEK293 cells were transfected with expression vectors using LipofectAMINE (Invitrogen) according to the manufacturer’s instructions.

**Immunostaining and fluorescence microscopy.** Cells were cultured on glass coverslips, fixed with 4% paraformaldehyde in PBS containing 0.1% MgCl2·6H2O, and permeabilized with 0.4% Triton X-100 in PBS for 5 min. To diminish nonspecific binding of the antibodies, the cells were incubated in 10% goat serum in PBS for 30 min. The cells were incubated with antibodies in PBS containing 10% goat serum, and then incubated with fluorescent Alexa 488-conjugated anti-rabbit IgG (Invitrogen) for 1 h. The cells were viewed directly with a 63× Plan-Neofluar oil immersion objective using a Zeiss confocal microscope (LSM5 Pascal).

**Biotinylation of cell surface proteins.** The cells were washed with ice-cold PBS and incubated with 0.5 mg/ml of sulfo-NHS-Biotin solution in PBS for 30 min on ice. They were washed with PBS to remove unbound sulfo-NHS-Biotin and lysed using RIPA buffer (20 mmTris–Cl (pH 7.5), 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate) containing protease inhibitors (100 μg/ml of (p-aminophenyl)mercurithiosulfon fluoride, 2 μg/ml leupeptin, and 2 μg/ml aprotinin). Immunopure Immobilized Mono-Meric Avidin Gel (Thermo Fisher Scientific) was added to the cell lysate to precipitate biotinylated proteins. The biotinylated proteins were electrophoresed on a 10% SDS-polyacrylamide gel and immunodetected.

**Western blotting.** The cells were washed with PBS and lysed in lysis buffer (50 mmTris–Cl (pH 7.5), 150 mmNaCl, and 1% Triton X-100) containing protease inhibitors (100 μg/ml of (p-aminophenyl)mercurithiosulfon fluoride, 2 μg/ml leupeptin, and 2 μg/ml aprotinin). Samples were electrophoresed on 10% SDS-polyacrylamide gel and immunodetected with antibodies.

**Results**

**Subcellular localization of ABCG proteins**

First, the subcellular localization of ABCG proteins was examined in HEK293 cells. Heterologously expressed ABCG proteins were detected with antibodies against the proteins by immunofluorescence analysis. As reported previously, ABCG1 and ABCG2 localized to the plasma membrane (Fig. 1). ABCG4 also localized to the plasma membrane. However, ABCG5 and ABCG8, when they were expressed alone, localized to the ER. When ABCG5 and ABCG8 were expressed together, they localized to the plasma membrane and ER. To confirm the localization of ABCG5 and ABCG8 to the plasma membrane, the cells were treated with membrane-impermeable sulfo-NHS-biotin, and biotinylated cell surface proteins were precipitated with avidin agarose (Fig. 2). ABCG1, ABCG2, and ABCG4 were biotinylated, whereas ABCG5 and ABCG8 were not, indicating that ABCG5 and ABCG8 did not reach the plasma membrane when expressed alone.

Next, the plasma membrane localization of ABCG5 and ABCG8 after coexpression was examined by biotinylation (Fig. 3). When ABCG5 and ABCG8 were coexpressed, bands of higher molecular weight were also detected by western blotting (Fig. 3, lower panels, indicated by asterisks). ABCG5 and ABCG8 in the higher-molecular-weight bands were biotinylated (Fig. 3, upper panels), suggesting that they were localized to the plasma membrane. In addition, they were resistant to endoglycosidase H digestion (data not shown), suggesting that they were modified with complex-type N-linked oligosaccharides. ABCG5 and ABCG8 in the lower-molecular-weight bands were immature forms modified with high mannose-type oligosaccharides because they were sensitive to endoglycosidase H digestion (data not shown), as reported previously. Although precipitation of immature ABCG5 by avidin agarose was scarcely observed as in Fig. 2, the precipitated band of the immature form of ABCG5 was detected as in Fig. 3. This was due to long exposure to show the biotinylated ABCG5. The immature form of ABCG5 appeared to interact with avidin agarose nonspecifically, because precipitation of the immature form of ABCG5 did not depend on biotinylation.

**Dimer formation of ABCG proteins**

To examine dimer formation, ABCG proteins were crosslinked with DSS or BS (Fig. 4). DSS and BS are membrane permeable and impermeable crosslinkers respectively, with arm-lengths of 11.4 angstroms. Bands around 130–150 kDa were detected when ABCG proteins were crosslinked with DSS, indicating that the ABCG proteins formed dimers. Higher molecular-
weight bands were also detected. When ABCG proteins were crosslinked with BS, dimer bands of ABCG1, ABCG2, and ABCG4 were detected, indicating that ABCG1, ABCG2, and ABCG4 were localized to the plasma membrane as homodimers.

Neither ABCG5 nor ABCG8 was crosslinked by BS.
when expressed alone (Fig. 5). However, they were crosslinked when expressed together. The dimers, crosslinked by BS, were detected by both anti-HA and anti-myc antibodies, suggesting that the heterodimer was predominantly translocated to the plasma membrane. These findings suggest that the heterodimer of ABCG5 and ABCG8 localized to the plasma membrane, whereas their homodimers were retained in the ER.

**Subcellular localization of ABCG chimeric proteins**

To identify the region responsible for ER retention of the homodimers of ABCG5 and ABCG8, plasmids to express chimeric proteins of ABCG1 and ABCG5 and of ABCG2 and ABCG8 were constructed (Fig. 6). The chimeric protein composed of the N-terminal cytosolic NBD of ABCG1 and the TMD of ABCG5 was named ABCG1-5. Plasmids to express chimeric proteins ABCG5-1, ABCG2-8, and ABCG8-2 were constructed likewise, and their subcellular localizations were examined. ABCG1-5 localized to the plasma membrane as did ABCG1, whereas ABCG5-1 localized to the ER similarly to ABCG5 (Fig. 7A). ABCG2-8 localized to the plasma membrane like ABCG2, whereas ABCG8-2 localized to the ER similarly to ABCG8 (Fig. 7B). To verify expression on the plasma membrane, a biotinylation assay was performed. ABCG1-5 was biotinylated and precipitated as ABCG1, whereas ABCG5-1 was not like ABCG5, suggesting that ABCG1-5 localized to the cell surface (Fig. 8). ABCG2-8 was biotinylated and precipitated, whereas ABCG8-2 was not (data not shown). These findings indicate that chimeric proteins with the cytosolic domain of ABCG1 or ABCG2 localized to the plasma membrane and suggest that the cytosolic domains of ABCG5 and ABCG8 are responsible for ER retention.

**Dimer formation of ABCG chimeric proteins**

To examine dimer formation of the chimeric proteins, they were crosslinked with DSS or BS (Fig. 9). ABCG1-5 was crosslinked by both DSS and BS, and a band corresponding to the dimer size was detected by western blotting as ABCG1. ABCG5-1 was crosslinked by DSS but not by BS, similarly to ABCG5. ABCG2-8 was crosslinked by both DSS and BS, whereas ABCG8-2 was crosslinked by DSS but not by BS (data not shown). These results suggest that the chimeric proteins ABCG1-5 and ABCG2-8 localize to the cell surface as homodimers.
**Fig. 6.** Schematic Representation of Chimeric Proteins.

ABCG1-5 chimeric protein has the N-terminal cytosolic nucleotide-binding domain (NBD) of ABCG1 and the C-terminal transmembrane domain (TMD) of ABCG5. ABCG5-1 has the N-terminal NBD of ABCG5 and the C-terminal TMD of ABCG1. ABCG2-8 has the N-terminal NBD of ABCG2 and the C-terminal TMD of ABCG8. ABCG8-2 has the N-terminal NBD of ABCG8 and the C-terminal TMD of ABCG2.

**Fig. 7.** Subcellular Localization of ABCG Chimeric Proteins.

HEK293 cells were transfected with ABCG1-FLAG, ABCG1-5-FLAG, ABCG5-1-FLAG, ABCG5-FLAG, myc-ABCG2, myc-ABCG2-8-FLAG, ABCG8-2, or ABCG8-FLAG. At 48 h after transfection, the cells were permeabilized using Triton X-100 and reacted with rabbit polyclonal anti-FLAG antibody (A), anti-mouse monoclonal myc antibody (ABCG2 and ABCG2-8), or rabbit polyclonal anti-ABCG8 antibody (ABCG8-2 and ABCG8) (B) and Alexa-488 conjugated secondary antibodies.
Effects of mutations in RXR-motif like sequence

The cytoplasmic domains of ABCG5 and ABCG8 do not contain known ER-retention signal sequences, such as C-terminal RRXX or KKXX. The formation of several membrane protein complexes, including ATP-sensitive potassium channels (K\textsubscript{ATP} channels), NMDA receptor, and GABA\textsubscript{B} receptor, is regulated by an ER-retrieval motif, the RXR (Arg-X-Arg) motif, located in the cytoplasmic region.\textsuperscript{19,20} The RXR motif is also involved in the trafficking of CFTR.\textsuperscript{21} It was assumed that this motif might be involved in ER retention of ABCG5 and ABCG8, because ABCG5 and ABCG8 contain two RXR sequences each (residues 198-RRR-200 and 241-RNR-243 in ABCG5, and 196-RLR-198 and 218-RRR-220 in ABCG8), which are conserved between human and mouse. An arginine residue in the RXR sequences was mutated to alanine, and the mutant ABCG5 and ABCG8 proteins were expressed in HEK293 cells. Immunofluorescence analysis indicated that each of the mutants localized to the ER when expressed alone and localized to plasma membrane when expressed with its counterpart (data not shown). The subcellular localization of the mutants was indistinguishable from that of the wildtype, indicating that the RXR sequences are unlikely to be ER-retrieval motifs for ABCG5 and ABCG8.

Discussion

This study analyzed the subcellular localization of ABCG proteins and their chimeric proteins. The homodimers of ABCG1, ABCG2, and ABCG4 and the heterodimer of ABCG5 and ABCG8 localized to the plasma membrane, whereas the homodimers of both ABCG5 and ABCG8 were retained in the ER. The homodimers of chimeric proteins ABCG1-5 and ABCG2-8 localized to the plasma membrane, whereas those of ABCG5-1 and ABCG8-2 were retained in the ER. These findings suggest that the cytoplasmic NBDs of ABCG5 and ABCG8 are responsible for ER retention of their homodimers. This is consistent with a study showing that the C-termini of ABCG5 and ABCG8 are not involved in the ER retention of their homodimers.\textsuperscript{22} Because mutations in the RXR sequences did not affect the subcellular localization of ABCG5 or ABCG8 and there are no known ER-retention or -retrieval motifs in the cytoplasmic domains of ABCG5 or ABCG8, novel motifs may exist within these domains.

It has been reported that the heterodimer of ABCG5 and ABCG8 is stable, whereas the homodimer was retained in ER and degraded with a half life of 3 h by proteasomes.\textsuperscript{15,18} Quality control in the ER is important not only in monitoring misfolded proteins but also in
maintaining a balance with regard to subunit synthesis. Several membrane proteins that function as a complex of multiple subunits are retained in the ER until they form a complete complex. For example, ABCC8 (SUR1), ABCC9 (SUR2), and Kir6.1x, subunits of KATP channels, contain ER-retention (RXXR) motifs in their sequences so that they are retained in the ER until they are incorporated into the complete complex.19) Once SUR and Kir form a complete heterotramer, they move from the ER to the plasma membrane and carry out their function. Otherwise, they are retained in the ER and degraded rapidly.23) The physiological significance of the fact that the homodimers of ABCG5 and ABCG8 are retained in the ER and are removed rapidly in ER-associated degradation processes is not clear. When ABCG5 and ABCG8 were coexpressed in HEK293 cells, only parts of ABCG5 and ABCG8 localized to the plasma membrane, as shown in Figs. 3 and 5. We speculate that reservation of monomers or homodimers of ABCG5 and ABCG8 in the ER is important in coping with sterol accumulation quickly, and that heterodimerization is regulated by sterol concentrations in the cells. Elucidating the molecular mechanisms of the membrane trafficking of ABCG5 and ABCG8 is important to understand the regulation of sterol transport by ABCG5/ABCG8.

It has been reported that not only ER-retention signals but also anterograde signals are involved in the quality control of K+TP channels.19,24) Further analysis of the subcellular localization of ABCG proteins might suggest that the cytoplasmic domains of ABCG1 and ABCG2 contain ER-export signals. ABCG5 and ABCG8 might also contain ER-export signals in addition to ER-retention signals, and membrane trafficking of ABCG5 and ABCG8 might be regulated by the balance between these two signals. When ABCG5 and ABCG8 form homodimers or a heterodimer, ER-retention and ER-export signals may work, respectively.

Oligomers of ABCG proteins were detected in the crosslinking experiment. It has been reported that ABCG2 exists mainly as a homotetramer.25) It is not clear whether the oligomer of ABCG proteins is functional, but the dimer form appeared to be dominant when the ABCG proteins were crosslinked with DSS and with BS in this study, and with DSG, DSP, and DTBP in previous studies.2,22) Okiyoneda et al. reported that ABCG5 was precipitated by avidin agarose after biotinylation, and suggested that part of ABCG5 was expressed in the plasma membrane. However, as shown in Fig. 3, ABCG5, modified with mannose-type N-linked oligosaccharide, was precipitated even without biotinylation, suggesting that a monomer or a homodimer of ABCG5 can interact with avidin agarose nonspecifically.

Both ABCG1-5 and ABCG1-1 formed homodimers, as shown by the crosslinking experiments. This suggests that the intact ABCG protein is not essential for dimer formation, and that the cytoplasmic or the transmembrane domain is involved in dimer formation. Further studies using chimeric proteins are needed to determine the region responsible for dimer formation.

In summary, this study indicates that the cytoplasmic domains of ABCG5 and ABCG8 are responsible for the ER retention of homodimers, and that known ER-retention or -retrieval motifs do not exist in the cytoplasmic domains. These results suggest that novel ER-retention or -retrieval motifs exist in the cytoplasmic domains of ABCG5 and ABCG8.

Acknowledgments

This work was supported by a Grant-in-aid for Scientific research (S) and a Grant-in-aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and by the Program for Promotion of Fundamental Studies in the Health Sciences of the National Institute of Biomedical Innovation of Japan. It was also supported by the World Premier International Research Center Initiative (WPI initiative), MEXT Japan.

References

12) Yu, L., Li-Hawkins, J., Hammer, R. E., Berge, K. E., Horton, J. D., Cohen, J. C., and Hobbs, H. H., Overexpression of


