ATP binding cassette transporter G1 (ABCG1) is an intracellular sterol transporter

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Four members of the mammalian ATP binding cassette (ABC) transporter G subfamily are thought to be involved in transmembrane (TM) transport of sterols. The residues responsible for this transport are unknown. The mechanism of action of ABCG1 is controversial and it has been proposed to act at the plasma membrane to facilitate the efflux of cellular sterols to exogenous high-density lipoprotein (HDL). Here we show that ABCG1 function is dependent on localization to intracellular endosomes. Importantly, localization to the endosome pathway distinguishes ABCG1 and/or ABCG4 from all other mammalian members of this superfamily, including other sterol transporters. We have identified critical residues within the TM domains of ABCG1 that are both essential for sterol transport and conserved in some other members of the ABCG subfamily and/or the insulin-induced gene 2 (INSIG-2). Our conclusions are based on studies in which (i) biotinylation of peritoneal macrophages showed that endogenous ABCG1 is intracellular and undetectable at the cell surface, (ii) a chimeric protein containing the TM of ABCG1 and the cytoplasmic domains of the nonsterol transporter ABCG2 is both targeted to endosomes and functional, and (iii) ABCG1 colocalizes with multiple proteins that mark late endosomes and recycling endosomes. Mutagenesis studies identify critical residues in the TM domains that are important for ABCG1 to alter sterol efflux, induce sterol regulatory element binding protein-2 (SREBP-2) processing, and selectively attenuate the oxysterol-mediated repression of SREBP-2 processing. Our data demonstrate that ABCG1 is an intracellular sterol transporter that localizes to endocytic vesicles to facilitate the redistribution of specific intracellular sterols away from the endoplasmic reticulum (ER).

Results

ABCG1 Localizes to Endosomes. To determine whether endogenous ABCG1 is expressed at the cell surface, we incubated primary mouse peritoneal macrophages for 24 h in the absence or presence of the LXR agonist GW3965. Cells were exposed to biotin before quenching the reaction to prevent modification of intracellular proteins after cell lysis. As expected, Western blot analysis of whole cell lysates showed that GW3965 markedly induced ABCG1 and ABCA1 protein levels, but had no effect on pan-cadherin or protein disulfide isomerase (PDI), markers for the cell surface and endoplasmic reticulum (ER), respectively (Fig. 1, compare lanes 1 and 2).

As expected, Fig. 1 (lanes 3 and 4) shows that ABCA1 and pan-cadherin are biotinylated and precipitated by the streptavidin beads, consistent with the known expression of both proteins at the cell surface. In contrast, ABCG1 and PDI were not.

These data suggest that, unlike ABCA1, which efuxes cholesterol and phospholipid after binding exogenous lipid-poor apoproteins, ABCG1 efuxes cellular cholesterol by a process that is not dependent upon interaction with an extracellular protein.

Studies with Abcg1−/−, Abcg1−/−/Abcg4−/−, Abcg1−/−/Apoe−/−, and Abcg1−/−/Abca1−/− mice indicate that loss of ABCG1 has no effect on plasma lipoprotein levels (8, 14–18). Rather, studies indicate that loss of ABCG1 was associated with an inability to control cellular sterol levels, especially in pulmonary macrophages (19, 20). This led to an alternative proposal that the primary function of ABCG1 may be to control intracellular sterol homeostasis, rather than to efux cellular sterols to HDL (21, 22).

Identification of the cellular location and substrates of ABCG1 might provide important insights into the normal physiologic function of ABCG1. However, there have been conflicting reports on the cellular localization of ABCG1 as it has been reported to be predominantly localized to the cell surface (23, 24) or to be transported to the plasma membrane in response to liver X receptor (LXR) agonists (25) or independent of LXR activation (24). Others have reported that ABCG1 is largely intracellular with little (21) or undetectable levels at the cell surface (22). The reasons for these discrepant conclusions remain unclear.

In the current studies, we use multiple approaches to demonstrate that ABCG1 is associated with endosomal vesicles and is undetectable at the cell surface. We also generated ABCG1–ABCG2 chimeric fusion proteins and demonstrated that the TM domains of ABCG1 are necessary and sufficient to target ABCG1 to the endocytic pathway and to increase the processing of SREBP-2, presumably by facilitating the movement of sterols away from the ER. Finally, we used a luciferase reporter assay to identify specific amino acids within the TM domains of ABCG1 that are necessary for function, but not for correct membrane targeting.
We previously reported that overexpression in primary mouse astrocytes of either ABCG1, or the highly homologous ABCG4, resulted in increased processing of the precursor SREBP-2 to form the nuclear/mature SREBP-2 (mSREBP-2) and to increased expression of SREBP-2-regulated genes that included the low density lipoprotein receptor (LDLR) and 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (Hmgcr) (22). In contrast, expression levels of many SREBP-2 responsive genes were repressed in the brains of Abcg1<sup>−/−</sup>, Abcg4<sup>−/−</sup>, or Abcg1<sup>−/−</sup>Abcg4<sup>−/−</sup> mice (15) or in macrophages from the lungs of Abcg1<sup>−/−</sup> mice consistent with intracellular accumulation of sterols and reduced processing of SREBP-2 (19). Given that small changes in ER sterol content are known to affect maturation/nuclear localization of SREBP-2 and subsequent target gene activation (20), these data suggest that ABCG1 may facilitate the movement of sterols away from the ER, thus increasing SREBP-2 processing. 

To test this hypothesis, we transfected CHO-K1 cells with a plasmid that encodes a luciferase reporter gene under the control of two SREs from the HMG-CoA synthase promoter (pSynSRE), and is thus activated by mSREBP-2, together with wild-type or epitope-tagged ABCG1, insulin-induced gene 2 (INSIG-2), SREBP cleavage-activating protein (SCAP), or various other ABC transporters. The activity of the luciferase reporter plasmid is completely dependent on endogenous SREBP processing and nuclear localization of mSREBP-2. Such processing of SREBP-2 is partially inhibited when cells are grown in the presence of 10% FBS (Fig. 3 A and B, empty plasmid). Overexpression of INSIG-2 and SCAP were used as controls as they are known to repress or induce SREBP-2 maturation, respectively (27, 28).

Fig. 3A shows that overexpression of ABCG1, ABCG4, or SCAP resulted in an approximately fourfold increase in luciferase activity, consistent with increased levels of mSREBP-2 and activation of target genes. Activation of pSynSRE by ABCG1, ABCG4, or SCAP was specific because luciferase activity was unchanged after overexpression of ABCG2, ABCA1, or ABCG5:ABC8, and, as expected (27), decreased after overexpression of INSIG-2 (Fig. 3A). Fig. 3B shows that the ability of ABCG1 to activate pSynSRE was significantly impaired when three FLAG epitopes or GFP were fused to the COOH terminus. In contrast, ABCG1 containing a single FLAG epitope was nearly as potent as untagged ABCG1 protein (Fig. 3B). All these data suggest that ABCG1 facilitates the movement of sterols away from the ER leading to increased nuclear mSREBP-2 and activation of target genes.

Finally, we show that, whereas overexpression of untagged ABCG1 or ABCG1–1×FLAG stimulated the efflux of radioactive cellular cholesterol to exogenous HDL, addition of larger tags
(3xFLAG or GFP) to ABCG1 severely impaired function (Fig. 3C). Importantly, comparison of the data in Fig. 3A and B vs. C, indicate that the luciferase reporter assay is far more robust and sensitive than the cholesterol efflux assay. As described below, this assay has led to the identification of critical amino acids required for ABCG1 function.

Transmembrane Domains of ABCG1 Are Sufficient for Endosomal Targeting and Sterol Transport. To identify domains of ABCG1 necessary for intracellular targeting and function, we generated plasmids that expressed wild-type ABCG1 or ABCG2 or chimeric fusion proteins comprising the NH2-terminal cytoplasmic domain (~440 residues) and TM domain (~220 residues) of ABCG1 and/or ABCG2, each with a single FLAG epitope at the COOH terminal (Fig. 4A).

The data of Fig. 4A (Upper Left, white arrows) show that overexpressed ABCG1 localizes to intracellular vesicles and is undetectable at the cell surface. The majority of ABCG2 localized to the plasma membrane (Fig. 4A; Upper Right, red arrows) where it can function to efflux drugs from the cell. Overexpression of ABCG1, but not ABCG2, increased both efflux of cellular cholesterol to HDL and the activity of pSynSRE (Fig. 4A and B). A chimeric fusion protein containing the NH2-terminal domain of ABCG2 and the TM domains of ABCG1 (ABCG2–ABCG1) localized to intracellular vesicles (Fig. 4A; Lower Right, white arrows). Importantly, this fusion protein retained significant functional activity as determined by the activation of pSynSRE and increased efflux of cellular cholesterol to HDL (Fig. 4A and B). In contrast, a chimeric fusion protein containing the NH2-terminal domain of ABCG1 and the TM domains of ABCG2 (ABCG1–ABCG2) localized to the cell surface (Fig. 4A, Lower Left, red arrows) and failed to activate pSynSRE or the efflux of cellular cholesterol to HDL (Fig. 4A and B). Taken together, these data demonstrate that the TM domains of ABCG1 are sufficient to correctly target the protein to intracellular vesicles and to stimulate both pSynSRE luciferase activity and cellular cholesterol efflux to HDL and that ABCG1 must be localized to intracellular vesicles to function.

Identification of Critical Residues Required for ABCG1 Function. To identify critical residues that are required for ABCG1 to activate pSynSRE and/or the efflux of cellular cholesterol to HDL, we aligned the amino acid sequence of the TM domains of mouse ABCG1 with the corresponding sequences from three other members of the murine ABCG subfamily (ABCG4, ABCG5, and ABCG8) that are reported to be involved in sterol flux (Fig. S1). We included INSIG-2, as alanine-scanning mutagenesis studies previously identified five amino acids (asterisks in Fig. S1) that are important for INSIG-2 to bind oxysterols and thus retain the SCAP:SREBP complex in the ER (29). We also included ABCG2 as a negative control because this protein does not affect sterol homeostasis or efflux of cellular sterols (Fig. S1).

We excluded proteins that contained a sterol-sensing domain (SSD) (i.e., SCAP, NPC-1, HMG-CoA reductase) (30–32) because comparison of their amino acid sequences with ABCG1 failed to identify significant regions of amino acid conservation (Fig. S2A and B).

On the basis of the alignment (Fig. S1), we generated a series of ABCG1 mutants wherein a single amino acid was replaced with alanine. We chose to mutate two residues (Q498A and W511A) that are both conserved with INSIG-2 and were previously shown to be important for the interaction of INSIG-2 with oxysterols (29). We also mutated residues that were either conserved with INSIG-2 and at least two other ABCG family members (T513A, G569A, L534A, and L541A) or were found in at least two members of the ABCG subfamily but not INSIG-2 (G422A, Y479A, F455A, and S573A). The positions of the 10 amino acid mutations within the six TM α-helices and the point mutation in the Walker A motif are illustrated in Fig. S3.

CHO-K1 cells were then transfectected with untagged wild-type or mutant ABCG1 together with pSynSRE. The data of Fig. 5 show that luciferase activity increased 5.5- to 8-fold after overexpression of wild-type ABCG1 or ABCG1 mutants G422A or S573A, thus indicating that G422 and S573 are not critical for function. In contrast, luciferase activity did not increase significantly following overexpression of ABCG1–K124M that contains a mutation in a critical lysine in the Walker A motif (Fig. 5). This mutant ABCG1 serves as a negative control as

![Fig. 4. The C-terminal transmembrane domain region of ABCG1 is essential for cellular localization and function. (A) Cos-7 cells were transfected with 1xFLAG-tagged ABCG1, ABCG2, or the indicated chimeric fusion proteins. Confocal images (taken at 63x magnification) are shown. White and red arrows identify immunofluorescent signals in endosomes and the plasma membrane, respectively. (B) CHO-K1 cells were transfected with an empty/control plasmid or plasmids encoding the indicated protein and cholesterol efflux to HDL determined as described in Fig. 3. (C) CHO-K1 cells were cotransfected with pSynSRE and the indicated plasmid before determination of luciferase activity, as described in Fig. 3. Data in B and C are representative of two to three different experiments, each performed in sextuplet. *P < 0.05; ***P < 0.001 vs. cells transfected with pSynSRE and empty plasmid.](image-url)
mutation of the corresponding lysine in the Walker A motif of many other ABC transporters attenuates ATP hydrolysis and transport function (33). Interestingly, overexpression of the remaining eight ABCG1 constructs failed to induce luciferase activity above the level seen with the ABCG1–K124M mutant (Fig. 5). The finding that S573 is only three residues from G569, a nonfunctional mutant, suggests the effects of the mutations are specific. Thus, we conclude that these eight conserved residues are critical for maintaining ABCG1 function.

Western blot analysis of cell lysates showed that wild-type and mutant forms of ABCG1 were all expressed at similar levels (Fig. 5, Inset). Further, immunofluorescence studies of selected mutants showed that wild-type and inactive mutant ABCG1 proteins colocalized with NPC-1 (Fig. S4), suggesting the single point mutations did not result in aberrant targeting or degradation of ABCG1.

To determine whether dimerization was affected by these point mutations, we transfected cells with ABCG1–FLAG together with wild-type or mutant forms of ABCG1 that contained a COOH-terminal HA epitope. Immunoprecipitation followed by Western blot analysis of the precipitated proteins demonstrated that all five of the tested mutant proteins formed dimers in HEK293 cells (Fig. S5). We conclude that the loss in activity of the eight mutant forms of ABCG1 (Fig. 5) is not a result of decreased protein expression, mislocalization, or an inability to form homodimers. Rather, the data suggest that these eight amino acids are critical for ABCG1 to function and alter intracellular sterol flux and SREBP-2 processing.

**Inhibition of SREBP-2 Processing by Exogenous Sterols Is Attenuated by ABCG1.** Previous studies by Brown and Goldstein and colleagues demonstrated that processing of SREBP-2 in cultured cells is inhibited following addition of specific sterols to the media (29). Further, Adams et al. demonstrated that exogenously delivered 25-hydroxycholesterol (25-OHC) was more potent than cholesterol in inhibiting SREBP-2 processing (34).

To determine whether overexpression of ABCG1 affects the ability of exogenous sterols to repress SREBP-2 processing, we transfected CHO-K1 cells with pSynSRE, together with either wild-type or mutant ABCG1. Cells were incubated for an additional 24 h in medium supplemented with 5% lipoprotein-deficient serum, 5 μM simvastatin and 50 μM mevalonic acid to activate SREBP-2 processing and induce SREBP-2 target genes, including pSynSRE. As expected, under these conditions overexpression of ABCG1 had a relatively small effect on luciferase activity, consistent with preexisting high levels of nuclear mSREBP-2 (Fig. 6A, compare open bars).

Figure 6A (Left two bars) shows that treatment of the cells with cyclodextrin (CD):cholesterol (25 μM) reduced the activity of pSynSRE by 60%, consistent with the ability of cholesterol to inhibit SREBP-2 processing and mSREBP-2. Importantly, this cholesterol-dependent repression of luciferase activity was attenuated in cells overexpressing wild-type ABCG1, but not the inactive mutant ABCG1–K124M (Fig. 6A). We interpret these data to indicate that wild-type ABCG1 redistributes cholesterol away from the ER, thus allowing maturation of SREBP-2 and activation of pSynSRE. Figure 6B shows the changes in pSynSRE activity in response to different oxysterols (0.5 μM) in the presence or absence of either wild-type or mutant ABCG1 overexpression. The data show that repression of pSynSRE luciferase activity, in response to 27-hydroxysterol (27-OHC) and 25-OHC, was blunted by overexpression of wild-type, but not mutant ABCG1 (Fig. 6B). In contrast, repression of the reporter activity following addition of 7-ketocholesterol, an autooxidation product of cholesterol, to the cells was unaffected by ABCG1 overexpression (Fig. 6B). Addition of 7-β-cholesterol, another autooxidation, nonenzymatic product of cholesterol, resulted in relatively poor repression of pSynSRE and the effect was not impaired by ABCG1 overexpression (Fig. 6B). Finally, we show that the ability of transfected wild-type, but not mutant ABCG1 to limit the oxysterol-mediated repression of pSynSRE luciferase activity was dependent both on oxysterol concentration and structure (Fig. 6 C and D). The data show that wild type, but not mutant ABCG1 is able to blunt the repression of pSynSRE in response to 27-OHC at concentrations below 1 μM (Fig. 6C). In contrast, the ability of ABCG1 to attenuate repression of pSynSRE in response to 27-OHC was muted over the whole concentration range tested (Fig. 6D). The effect of ABCG1 overexpression on oxysterol-mediated inhibition of SREBP-2 processing is specific, as the data in Fig. 6C show that ABCG1 fails to affect the repression of pSynSRE in response to 7-ketocholesterol. Together, these studies demonstrate that ABCG1 modulates the biological effects of specific sterols (cholesterol, 27-OHC, and 25-OHC) on SREBP-2 processing and maturation.

**Discussion**

Mammalian ABC transporters have been shown to localize to either the plasma membrane, peroxisomes, mitochondria, endoplasmic reticulum (3–5), or lamellar bodies (6). Very recently Sturek et al. (21) showed that >90% ABCG1 was concentrated in secretory granules of pancreatic β-cells. These authors provided evidence that stimulated insulin secretion was dependent upon ABCG1 maintaining appropriate cholesterol levels in the granule membrane (21). To our knowledge, this is the only report demonstrating that a member of the ABC gene superfamily localizes to the endosome/recycling endosome pathway. The conclusion that active ABCG1 localizes to endosomes is supported by data obtained from a number of different experimental approaches, including biotinylation of endogenous ABCG1 in peritoneal macrophages treated with or without an LXR agonist (Fig. 1), immunofluorescent studies involving either untagged or epitope-tagged ABCG1, or hybrid ABCG1–ABCG2 proteins (Figs. 2 and 4), and the ability of the intracellular ABCG1 protein to activate an SRE-luciferase reporter gene (Figs. 3–6). This conclusion is also consistent with a previous observation showing that transiently transfected ABCG1 or ABCG4 localized to intracellular vesicles in primary mouse neurons and astrocytes and to RhôB5 vesicles in COS-7 cells (22). On the basis of all these data, we hypothesize that one important function of ABCG1 is to maintain normal sterol levels in endocytic vesicles in part by facilitating the flux of specific sterols away from the endoplasmic reticulum.

The current studies also identify important differences between ABCG1 and the highly homologous transporter ABCG4 (22) and other ABC family members that facilitate “sterol” transport. In contrast to the intracellular localization of ABCG1 and ABCG4 (22), both ABCA1 and the heterodimer ABCG5:ABCG8 localize to the plasma membrane (3, 35). Further, heterodimeric ABCG5:ABCG8 promotes the efflux of cholesterol.
and sitosterol out of enterocytes or hepatocytes (36), whereas ABCA1 may actually stimulate the transbilayer movement of a phospholipid, with sterol redistributing into the phospholipid-enriched outer leaflet of the plasma membrane, before efflux of both lipids to exogenous apoA-1 (37).

To our knowledge, there are no other reports of alanine-scanning mutagenesis of an ABC sterol transporter. This approach, together with studies using fusion proteins containing domains of ABCG1 and ABCG2, has allowed us to separate the requirements for correct intracellular targeting and function. We show that the TM domains of ABCG1 are sufficient to both target the protein to intracellular vesicles of the endosomal pathway and to alter cellular sterol homeostasis (Fig. 4). However, our mutagenesis studies have led to the identification of eight amino acids within the TM domain that are critical for function, but are themselves not important for ABCG1 targeting. Interestingly, two of the residues (Q498 and W511) required for ABCG1 function are conserved in INSIG-2, where they are involved in interaction of ABCG1 with specific oxysterols (27-OHC and 25-OHC) (Fig. 6).

On the basis of biotinylation of HEK293 cells that expressed ABCG1–FLAG in response to mifepristone (23) or studies using sucrose gradient centrifugation (25), it was concluded that ABCG1 localized to the plasma membrane. However, as we have never observed ABCG1 at the cell surface, we are unable to account for these differences.

The current studies were greatly aided by the use of a highly sensitive and robust sterol-sensitive luciferase reporter assay that was responsive to mSREBP levels. In contrast to the [3H]-cholesterol efflux assay to measure ABCG1 activity, the pSynSRE assay is more sensitive, reproducible, and can be carried out in normal growth media (10% FBS) and does not depend upon the addition of acyl-CoA:cholesterol acyltransferase (ACAT) inhibitors or culturing cells for prolonged periods in the absence of serum.

On the basis of recent studies that showed that a small decrease or increase in ER-cholesterol levels is sufficient to activate or repress SREBP-2 processing, respectively (26), we have concluded that ABCG1 functions to redistribute intracellular cholesterol and specific oxysterols (27-OHC and 25-OHC) (Fig. 6) away from the ER, thus leading to increased mSREBP-2 levels and activation of pSynSRE (Fig. 6). The finding that ABCG1 coexpression had no effect on the repression of pSynSRE by 7-ketocholesterol (Fig. 5) was unexpected, as it has been reported that ABCG1 protects cells from 7-ketocholesterol–induced toxicity (39). The reason for these differences remains to be identified.
Nonetheless, the observation that repression of SREBP-2 processing by 27- or 25-OHC is attenuated following overexpression of ABCG1 suggested that these oxysterols may accumulate in cells expressing this transporter. Consistent with this proposal, we have previously reported that ABCG1–alveolar macrophages accumulate significant amounts of 27-OHC and 25-OHC (17). Further, cholesterol 27-hydroxylase (Cyp27A1) and cholesterol 25-hydroxylase (CH25H) mRNAs, that encode the enzymes that synthesize these two oxysterols, were increased in ABCG1–macrophages (Fig. S7).

How can intracellular localization of ABCG1 affect efflux of cellular cholesterol to exogenous HDL? The finding that ABCG1 is present in endosomes and recycling endosomes suggests a mechanism by which ABCG1 transfers sterols to the inner leaflet of these vesicles before their fusion with the plasma membrane. This would result in redistribution of these sterols to the outer leaflet of the plasma membrane such that they can desorb in a nonspecific manner to multiple exogenous lipid acceptors that include HDL, LDL, or nonphysiologically to phospholipid vesicles or CD. Such nonspecific desorption of cholesterol from the plasma membrane of ABCG1-expressing cells has been recently reported (13). In summary, the studies described herein provide important insights into the domains and residues that are important for ABCG1 function and localization. Further studies that use purified ABCG1 protein should provide additional information on possible transporter–substrate interactions.

Materials and Methods

Materials. Details of materials can be found in SI Materials and Methods.

Preparation of Sterol-Methyl-β-Cyclodextrin Complexes. Complexes were generated using a modification of the protocol described by Klein et al. (40). Details can be found in SI Materials and Methods.

Further detailed methods can be found in the SI Materials and Methods.

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Supporting Information

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SI Materials and Methods

Materials. FuGene 6 and complete protease inhibitor mixture were from Roche Applied Science; Ni²⁺-NTA agarose beads from Qiagen; cholesterol; 7-ketocholesterol, mouse anti-His monoclonal antibody, and anti-FLAG M2 monoclonal antibody from Sigma; protein G PLUS-agarose, rabbit anti-PDI antibody and mouse anti-pan-cadherin antibody from Santa Cruz Biotechnology; ABCA1 and ABCG1 polyclonal antibodies from Novus Biologicals; Luciferase Reporter Assay system from Promega; CHO-K1 cells from ATCC (CCL-61); EZ-Link Sulfo-NHS-SS Biotin and NeutrAvidin agarose beads from Thermo Scientific; 25-hydroxycholesterol, 27-hydroxycholesterol and 7-β-hydroxycholesterol from Avanti Polar Lipids; methyl-β-cyclohexatin and hydroxypropyl-β-cyclohexatin from Trappol; H-cholesterol (60-90 Ci/mmol) from American Radiolabeled Chemicals; anti-rabbit and anti-mouse HRP-conjugated secondary antibodies from BioRad; and 4× SDS sample loading buffer from Invitrogen.

Buffers. Buffer A contained 10 mM Hepes-KOH pH 7.4, 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM sodium EGTA, 250 mM sucrose, 1% Triton X-100, and 1 protease inhibitor mixture tablet. PBS⁺⁺ contained 1x PBS supplemented with 0.02 mM CaCl₂ and 0.15 mM MgCl₂. Quenching buffer contained PBS⁺⁺ supplemented with 100 mM glycine. Cell lysis buffer (5×) contained 0.5 M Tris-phosphate (pH 7.8), 1 M DTT, 0.1 M CDTA, 50% (vol/vol) glycerol, and 5% (vol/vol) Triton X-100.

Culture Medium. Medium A contained Kainh’s modification of Ham’s F-12 medium supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin sulfate. Medium B comprised medium A supplemented with 10% FBS. Medium C comprised medium A supplemented with 0.2% BSA. Medium D was medium A supplemented with 5% LPDS, plus 5 μM simvastatin and 50 μM mevalonic acid. Medium E was DMEM supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin sulfate. Medium F comprised medium E supplemented with 10% FBS. Medium G comprised medium E supplemented with 5% LPDS, 5 μM simvastatin, and 50 μM mevalonic acid. Medium H comprised medium E supplemented with 0.2% BSA.

Plasmids. Full-length, untagged mouse ABCG1 (666 amino acids) was expressed in pcDNA3.1 under the control of a CMV promoter. Untagged ABCG1 containing single point mutations (alanine scanning) were generated by site-directed mutagenesis (QuikChange II XL kit; Stratagene). The coding regions of all ABCG1 alanine mutants were sequenced to confirm they contained no other mutations. For domain swapping experiments, full-length ABCG1 or ABCG2 containing a single COOH-terminal Flag epitope were cloned into pcDNA3.1. Fusion proteins were expressed from pcDNA3.1 and contained amino acids 1–409 of mouse ABCG1 fused to amino acids 394–655 of human ABCG2 (ABCG1-ABCG2), or amino acids 1–393 of human ABCG2 fused to amino acids 410–666 of mouse ABCG1 (ABCG2-ABCG1). These fusions proteins contained a single COOH-terminal Flag epitope. Where indicated, pcDNA3.1 contained wild-type mouse ABCG1 fused to either three tandem COOH-terminal Flag epitope tags or GFP. The cDNA for mouse Niemann-Pick type C-1 (NPC-1) (amino acids 1–1,277) was cloned into pEYFP-N1 to generate NPC-1 fused to YFP at the COOH terminus. The following recombinant expression plasmids have been previously described by others elsewhere: pTK-herpes simplex virus (HSV–SCAP–T7, encoding HSV fused to hamster SCAP under the control of the HSV-driven thymidine kinase (TK) promoter (1); pTK–INSIG–2–Myc, encoding human INSIG-2 fused to six tandem copies of a c-myc epitope tag driven by the HSV–TK promoter (2, 3); a sterol-sensitive luciferase reporter plasmid encoding a generic TATA box and three sterol response elements (SRE: −325 to −225) from the hamster 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase promoter fused into the luciferase pGL2 basic vector (pSynSRE) (4); pDsRed–Rab5 and pDsRed–Rab11 encoding human Rab5 and Rab11 fused to DsRed under the control of a CMV promoter (5). Detailed primer sequences are available upon request.

Preparation of Sterol/Methyl-β-Cyclohexatin Complexes. Cholesterol, and oxysterol derivatives of cholesterol, were complexed to cyclohexatin using a minor modification of the protocol described by Klein et al. (6). Briefly 10 mg of the indicated sterol (stock concentration 10 mg/mL) in 100% ethanol was added slowly to a stirred solution of 5% wt/vol methyl-β-cyclohexatin at 80 °C, until a clear solution was obtained. The resulting solution was lyophilized and the dried complex reconstituted in nanopure water to a sterol concentration of 2.5 mM.

Biotinylation of Cell Surface Proteins. Freshly isolated primary mouse peritoneal (thiglycocolate-elicited) macrophages were allowed to adhere to six-well plates for 18 h in medium F. Cells were treated in medium G with or without 1 μM liver X receptor (LXR) agonist GW3965 for 24 h. Cells were washed in PBS++ and then incubated for 30 min on ice with 250 μM EZ-link Sulfo-NHS-SS Biotin (diluted in PBS++). The cells were washed in PBS++ and the reaction was quenched for 30 min on ice in quenching buffer. Biotin-modified proteins were immunoprecipitated with NeutrAvidin streptavidin beads overnight at 4 °C. Biotin-modified proteins were collected by centrifugation at 5,000 × g for 5 min. Intracellular, unmodified proteins were collected from the supernatant of the 5,000 × g spin. The streptavidin beads were washed three times in PBS++ before proteins were removed from the beads by incubation at 42 °C for 20 min, in 2× SDS sample loading buffer supplemented with β-mercaptoethanol.

Immunoprecipitation of Protein Complexes. HEK293 cells were transfected in medium F with the indicated plasmids. Cells were lysed in buffer A. Proteins (100 μg) in buffer A were incubated overnight with anti-FLAG M2 antibody (used at 1:1,000) at 4 °C. Protein complexes were incubated with protein G-PLUS agarose beads at 4 °C for 6 h before centrifugation at 5,000 × g for 5 min. Beads were washed with buffer A. Protein complexes were retrieved by incubation at 42 °C for 20 min in 2× SDS sample loading buffer supplemented with β-mercaptoethanol.

Western Blot Analysis. From biotinylation studies, 5% of each fraction (total cell lysate, biotinylated proteins, unmodified intracellular proteins) was loaded and separated by SDS/PAGE. From immunoprecipitation studies, 20% of protein complexes were separated by SDS/PAGE. From overexpression and immunoprecipitation studies, cells were lysed in buffer A, and proteins (10 μg) were separated by SDS/PAGE. Proteins were transferred to polyvinylidene difluoride membrane. Primary antibodies were diluted 1:1,000 (ABCA1, ABCG1, pan-cadherin, HA) or 1:5,000 (PDI, β-actin) in 1× TBS containing 0.1% Tween 20 and 5% nonfat milk. Immune complexes were detected with anti-rabbit (ABCA1, ABCG1, PDI) or anti-
mouse (β-actin, pan-cadherin, HA) HRP-conjugated secondary antibodies diluted 1:10,000.

**Cell Transfection and Luciferase Reporter Assay.** CHO-K1 cells were plated in medium B 1 d before transfection and cultured to reach 80% confluence. Medium B was replaced with medium A before transfection. Cells were transfected using FuGeneHD (Roche) according to manufacturer instructions. Each well was transfected with 100 ng pSynSRE plus 5 ng of each expression plasmid, and 50 ng β-galactosidase expression plasmid as a control to normalize for minor changes in transfection efficiency. After 5 h, the culture medium was replaced with specific treatment media, as indicated.

Cells were cultured for 24 h, washed twice with PBS, and lysed with 1x cell lysis buffer. The luminometer assays were performed using Promega luciferase reporter assay system according to instructions. The luciferase activity was measured using a Centro LB 960 luminometer (Berthold Technologies).

**Cellular Efflux of Cholesterol.** HEK293 or CHO-K1 cells transfected with the indicated plasmids were incubated with 1 μCi/mL 3H-cholesterol for 24 h in medium H. The media was removed, the cells washed and equilibrated for 24 h in medium H. To determine cellular cholesterol efflux, cells were incubated in fresh medium H in the presence or absence of 50 μg/mL HDL for 4 h. The radioactive content of the cells and media was determined as previously described (7). Cholesterol efflux was determined by dividing the radioactive content of the media by the sum of the radioactivity in the cells and the media. The basal efflux (% cholesterol that effluxes to medium H; ~1–2%) was subtracted from the values obtained in the presence of HDL.

**mRNA Quantification.** Alveolar macrophages were isolated from bronchoalveolar lavage as previously described (8). Total RNA was isolated from alveolar macrophages using TRIzol (Invitrogen), and cDNA synthesized (Applied Biosystems). Quantitative real-time PCR was performed on a LightCycler 480 (Roche). mRNA expression was normalized to expression of 36B4. Detailed primer sequences are available upon request.

**Statistics.** Statistical analysis was performed using GraphPad Prism 5. For luciferase reporter assays and cholesterol efflux assays, statistical analysis was performed by one-way ANOVA, followed by a Tukey's multiple comparison test. For ABCG1 (wild-type, mutant, or chimera) transfected cells. For luciferase reporter assays with CD:sterol complex treatment, statistical analysis was performed by two-way ANOVA.

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**Fig. S1.** Alignment of members of the murine ABCG family with human INSIG-2 identifies conserved residues. Amino acids are numbered based on the murine ABCG1 sequence. TM 1–6 are boxed (dashed lines). Individual boxed residues (shaded gray) indicate some level of conservation. *Identifies residues of INSIG-2 that when mutated resulted in loss of INSIG-2 function (1).
A. Comparison of transmembrane (TM) α-helices 1–6 of ABCG1 with reported sterol-sensing domains. The sequence corresponding to TM 1–6 of ABCG1 was aligned and compared with the previously reported sterol-sensing domain of HMG-CoA reductase, SCAP, and NPC-1.

B. Schematic of an ABCG1 monomer showing the approximate location of individual amino acid mutations (black ovals) within the six TM α-helices (1–6) or the Walker A motif.
Fig. S4. Wild-type ABCG1 and ABCG1 containing inactivating alanine substitutions localize to similar intracellular vesicles. Cos-7 cells were cotransfected with plasmids encoding either untagged wild-type ABCG1 or the indicated mutant forms of ABCG1 and NPC-1–YFP. All images were taken at 63× magnification. Yellow dots indicate colocalization in the merged images.

Fig. S5. Wild-type ABCG1 and ABCG1 containing alanine substitutions form homodimers. HEK293 cells were cotransfected with wild-type (WT) ABCG1–FLAG and either WT ABCG1–HA or the indicated mutant ABCG1–HA. Cells were lysed and protein samples (10 μg) were analyzed by Western blot (Lower, immunoblot, IB). Cell lysates were also treated with anti-FLAG and the immunoprecipitated (IP) protein complexes separated by SDS/PAGE, transferred to a membrane, and the membrane probed with anti-HA (Upper).
**Fig. S6.** ABCG1 does not attenuate 7-ketocholesterol–mediated repression of SREBP-2 processing. CHO-K1 cells were transfected with pSynSRE, an empty plasmid (open bars) or a plasmid encoding either wild-type ABCG1 or ABCG1-K124M. Following 5 h transfection, cells were treated with the indicated concentration of oxysterol for 24 h before determination of normalized luciferase activity. **P < 0.01 vs. pSynSRE plus control.

**Fig. S7.** Alveolar macrophages from Abcg1<sup>−/−</sup> mice express elevated levels of the cholesterol modifying enzymes, 25- and 27-hydroxylase. Alveolar macrophages were isolated from the lungs of wild-type and Abcg1<sup>−/−</sup> mice. mRNA levels of cholesterol 25-hydroxylase (CH25-OH) and cholesterol 27-hydroxylase (CH27-OH) were measured by quantitative real-time PCR. mRNA levels were normalized to 36B4, and values given as fold change relative to wild-type mice. ***P < 0.001 vs. WT macrophages.