Intracellular Localization of Endogenous Mouse ABCG1 Is Mimicked by Both ABCG1-L550 and ABCG1-P550—Brief Report

Elizabeth J. Tarling, Peter A. Edwards

Objective—In a recent article in Arteriosclerosis, Thrombosis, and Vascular Biology, it was reported that ATP-binding cassette transporter G1 (ABCG1) containing leucine at position 550 (ABCG1-L550) was localized to the plasma membrane, whereas ABCG1-P550 (proline at position 550) was intracellular. Because the published data on the subcellular localization of ABCG1 are controversial, we performed additional experiments to determine the importance of leucine or proline at amino acid 550.

Approach and Results—We transfected multiple cell lines (CHO-K1, Cos-7, and HEK293 [human embryonic kidney]) with untagged or FLAG-tagged ABCG1 containing either leucine or proline at position 550. Immunofluorescence studies demonstrated that in all cases, ABCG1 localized to intracellular endosomal vesicles. We also show that both ABCG1-L550 and ABCG1-P550 are equally active in both promoting the efflux of cellular cholesterol to exogenous high-density lipoprotein and in inducing the activity of sterol regulatory element–binding protein-2, presumably as a result of redistributing intracellular sterols away from the endoplasmic reticulum. Importantly, we treated nontransfected primary peritoneal macrophages with a liver X receptor agonist and demonstrate, using immunofluorescence, that although endogenous ABCG1 localizes to intracellular endosomes, none was detectable at the cell surface/plasma membrane.

Conclusions—ABCG1, irrespective of either a leucine or proline at position 550, is an intracellular protein that localizes to vesicles of the endosomal pathway where it functions to mobilize sterols away from the endoplasmic reticulum and out of the cell. (Arterioscler Thromb Vasc Biol. 2016;36:1323-1327. DOI: 10.1161/ATVBAHA.116.307414.)

Key Words: ABC transporter ■ cholesterol homeostasis ■ lipids

Although the physiological functions of ATP-binding cassette transporter G1 (ABCG1) are currently unclear, there is overwhelming evidence to suggest that it is involved in the maintenance of tissue and cellular cholesterol homeostasis.1–7 ABCG1 is highly expressed in a wide variety of cells and tissues, including endothelial cells, lymphocytes, macrophages, B cells, pancreatic beta cells, neurons, and pulmonary epithelial cells.1–3,5–7,8,10 There is compelling evidence from ABCG1 loss-of-function mouse models to suggest that ABCG1 is required for normal function of many cell types.1–3,5–7,8,10 Whether all these changes are a result of altered control of intracellular sterol/lipid homeostasis remains unclear.

We previously reported that ABCG1 localized to intracellular vesicles of the endocytic pathway where it functions to control sterol flux away from the endoplasmic reticulum.11 We, and others, have also demonstrated that ABCG1 functions to promote the efflux of cellular sterols to various exogenous sterol acceptors, including high-density lipoprotein.5,6,8,11,12 It has also been reported that ABCG1 may transport specific phospholipids out of cells.13

In a recent paper, Gu et al14 suggested that the intracellular localization of ABCG1, as reported by Tarling and Edwards,11 was a result of a proline to leucine substitution at amino acid 550 of the mouse protein. Gu et al reported that although transfected mouse ABCG1 (mABCG1-L550) or human ABCG1 (hABCG1-L562) localized to the plasma membrane of HEK293 (human embryonic kidney) cells, substitution of proline at these positions led to intracellular localization.14 Nonetheless, cholesterol efflux from the transfected cells was reported to be independent of the leucine or proline at 550 of mouse or 562 of human ABCG1.14

Because the subcellular localization of ABCG1 has indeed been a controversial topic, we conducted an additional series of experiments with many different ABCG1 expression constructs, in multiple different cell lines, to determine the importance of leucine or proline at position 550 of the mABCG1 protein. Importantly, we also report on the localization of endogenous ABCG1 in primary mouse macrophages.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.
Results

Our aim was 2-fold: to review and use our comprehensive catalog of constructs of mABCG1 to determine whether a leucine or a proline at position 550 is important for the cellular localization of ABCG1 and to use select anti-ABCG1 antibodies to determine the cellular localization of endogenous ABCG1. We transfected both Cos-7 and CHO-K1 cells with empty plasmids or plasmids encoding untagged mABCG1 with either a leucine or a proline at position 550. We sequenced these plasmids and confirmed that the remaining amino acid sequence of ABCG1 corresponded to wild-type sequence reported by both NCBI (ncbi.nlm.nih.gov) and Ensembl (www.ensembl.org). The immunofluorescence data shown in Figure 1A are representative of numerous (>30) experiments. The data demonstrate that irrespective of whether there is a leucine or a proline at position 550, mABCG1 localizes to intracellular vesicles and is undetectable at the plasma membrane (Figure 1A; Figure 1A in the online-only Data Supplement). No signal was observed in cells transfected with an empty plasmid (Figure 1A). In addition, mABCG1-L550 and mABCG1-P550 both colocalize with NPC-1 (Niemann pick C type 1; Figure 1B), a marker of late endosomes, and with Rab5 and Rab11, markers for early and recycling endosomes, respectively (Figure IB and IC in the online-only Data Supplement). Further, both mABCG1-L550 and mABCG1-P550 were able to stimulate to a similar extent the efflux of radioactive cellular cholesterol from Cos-7 cells (Figure 1C) or CHO-K1 cells (Figure 1D) to exogenous high-density lipoprotein. Parallel immunocytochemistry studies performed after transfection of cells with epitope-tagged ABCG1-L550-FLAG or ABCG1-P550-FLAG indicated that in all cases, anti-FLAG antibodies identified ABCG1 in intracellular endosomes but failed to identify ABCG1 at the plasma membrane (Figure II in the online-only Data Supplement). In addition, colocalization studies in HEK293 cells transfected with either ABCG1-L550-FLAG or ABCG1-P550-FLAG demonstrated that ABCG1 localizes to intracellular vesicles and did not colocalize with the Na+/K+-ATPase protein, a plasma membrane marker (Figure III in the online-only Data Supplement). We previously described a sterol-sensitive luciferase reporter assay in which the luciferase activity was dependent on the processing of endogenous sterol regulatory element–binding protein-2. This assay is more robust and sensitive.

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**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ABCG1</td>
<td>ATP-binding cassette transporter G1</td>
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<td>LXR</td>
<td>liver X receptor</td>
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<td>mABCG1</td>
<td>mouse ATP-binding cassette transporter G1</td>
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Figure 1. mABCG1-L550 and mABCG1-P550 are intracellular and localize to endosomal vesicles. Cos-7 (A and B) and CHO-K1 (A) cells were transfected with either a control (empty) plasmid or a plasmid expressing untagged ABCG1 (A) or cotransfected with NPC-1 (Niemann pick C type 1)-YFP (B). Immunofluorescence was determined after incubation of cells with antibody to ABCG1 (Novus Biologicals, Lot E2). Images are at 63× magnification. Representative sites of colocalization are indicated by yellow arrows in the merged images (B, right). C and D, Cos-7 (C) and CHO-K1 (D) cells were transfected with either a control plasmid or a plasmid expressing untagged ABCG1-L550 or ABCG1-P550. Efflux of [1H] cholesterol to high-density lipoprotein (HDL; 50 μg/mL) was measured as described.2,4,11 E, CHO-K1 cells were transfected with a control plasmid or a plasmid expressing ABCG1-L550 or ABCG1-P550 together with pSynSRE and β-galactosidase. After 24 hours, the luciferase activity was determined as described.11 The data (presented as mean luciferase activity [fold change] or cholesterol efflux [%] ±SEM) of panels. C–E are representative of at least 3 separate experiments, each performed in quadruplicate (C and D) or sextuplet (E). ***P<0.001. ABCG1 indicates mouse ATP-binding cassette transporter G1; and mABCG1, mouse ATP-binding cassette transporter G1. (Downloaded from http://atvb.ahajournals.org by guest on November 10, 2017)
than the cholesterol efflux assay and is sensitive to the level of sterols in the endoplasmic reticulum that control sterol regulatory element–binding protein-2 maturation. We now demonstrate that overexpression of either mABCG1-L550 or mABCG1-P550 resulted in an ≈4-fold increase in luciferase activity (Figure 1E). Taken together, these data demonstrate that transfected mABCG1-L550 and mABCG1–P550 both localize to endocytic vesicles, and both are equally active in promoting cholesterol efflux to high-density lipoprotein or activating sterol regulatory element–binding protein-2 processing. In contrast to the studies of Gu et al., we obtained no evidence that mABCG1-L550 or mABCG1-P550 localized to the cell surface/plasma membrane under the conditions used here.

Antibodies to ABCG1 have almost universally lacked the specificity required for immunolocalization studies of the endogenous protein in cells or tissues. We obtained one particular lot of anti-ABCG1 that exhibited unusually high specificity. Resident peritoneal macrophages were isolated at the same time from both wild-type and Abcg1–/– littermate mice. The cells were plated on cover slips and incubated under identical conditions (media containing 10% fetal calf serum) for 24 hours and then treated for an additional 24 hours with vehicle or the liver X receptor (LXR) agonist GW3965 to induce ABCG1. We then performed immunohistochemistry in parallel and under exactly the same conditions. The immunofluorescence signal in the Abcg1–/– cells was extremely faint and unaffected by the GW3965 treatment, consistent with a high degree of specificity of this antibody (Figure 2A, lower panels). In contrast, Figure 2A (open arrows) shows that the vehicle-treated wild-type cells stained positive for ABCG1 and that the signal increased after incubation with the LXR agonist (closed arrows). Indeed, quantification demonstrated that the fluorescent signal increased over 5-fold when wild-type cells were treated with the LXR agonist (Figure 2B). Importantly, there was no change in cellular localization in response to treatment with the LXR agonist (Figure 2A, upper panels). In contrast, there was no change in the low signal derived from Abcg1–/– cells (Figure 2B). Thus, the data from these immunofluorescence studies indicate that endogenous ABCG1 is localized to intracellular vesicles of primary mouse peritoneal macrophages. Evidence that GW3965 treatment increased ABCG1 protein levels of peritoneal

Figure 2. Endogenous mouse ATP-binding cassette transporter G1 (ABCG1) is intracellular. A–D. Freshly isolated mouse resident primary peritoneal macrophages were isolated in 8 mL Dulbecco’s Modified Eagle’s Medium (DMEM) and plated in DMEM containing 10% FBS. After 20 hours, cells were treated with or without liver X receptors (LXR) agonist GW3965 (1 μM) for 24 hours before either fixing in 4% paraformaldehyde (A) as previously described or being harvested in 1× RIPA buffer containing protease inhibitor cocktail and ALLN calpain inhibitor (25 μg/mL; C and D). A, Resident peritoneal macrophages were incubated with antibody to ABCG1 (Novus Biologicals, Lot E2). Punctate intracellular organelles are indicated by white arrows. All fields show 4 to 7 macrophages. A total number of n=45 cells were counted per condition across 6 fields. B, Relative fluorescence was determined using ImageJ software (NIH). Data are presented as mean relative fluorescence±SEM. ***P<0.001. C and D, Western blot analysis of wild-type (C) and Abcg1–/– (D) resident peritoneal macrophages (total cell lysates), treated as in (A).
macrophages ≈3-fold and was easily detected with ABCG1 antisera is shown in Figure 2C. We also assessed the specificity of the antibody on Western blots using peritoneal macrophages isolated from Abcg1−/− mice treated with either vehicle or GW3965 as shown in Figure 2A and 2C. No signal for ABCG1 protein (≈72 kDa) was observed in Abcg1−/− cells, consistent with high specificity of the antisera for ABCG1 (Figure 2C). These data are consistent with the proposal that although endogenous ABCG1 is undetectable at the cell surface, it is detectable at intracellular locations.

Discussion

Understanding both the cellular localization of ABCG1, together with identification of critical amino acids and domains, will provide essential insight into the physiological function of ABCG1 in lipid transport. We have previously used alanine scanning to identify critical amino acids in the transmembrane domains of ABCG1 that are required for sterol transport function and localization.11 In contrast to the findings of Gu et al,14 but consistent with our previous published studies using many cell types,1,4,9,11,15 we unequivocally show, using multiple cell lines, that ABCG1 localizes to intracellular endosomal vesicles, whereas it is undetectable at the plasma membrane regardless of whether ABCG1 contains L550 or P550 (Figure 1A and 2B; Figures I–III in the online-only Data Supplement). We also show, using 2 independent assays, that both mABCG1-L550 and mABCG1-P550 are equally active in functioning to redistribute intracellular sterols and to promoting the efflux of cellular cholesterol to exogenous high-density lipoprotein (Figure 1C and 1D). Consequently, based on our current and earlier extensive studies, we are unable to explain the findings of Gu et al,14 wherein mABCG1-L550 was reported to localize to the plasma membrane of rounded cells, whereas mABCG1-P550 was shown to be found at both the plasma membrane and intracellular sites.

One alternative explanation for the different findings would depend on the specificity of the antibody used in immunofluorescence studies. The antibody used in the current, and our earlier studies, is from Novus Biologicals. Most lots of this Novus antibody identify a protein of ≈72 kDa on Western blots but fail to identify the ≈72 kDa protein in cells lacking ABCG1 (Figure 2C). However, only one lot of anti-ABCG1 (Lot E2) from Novus Biologicals that we have tested exhibited a differential signal when used in immunofluorescence studies using wild-type and Abcg1−/− cells (Figure 2A). In contrast, no data are provided in the study by Gu et al for Western blots or immunofluorescence studies using anti-ABCG1 H-65 in cells or tissues that lack ABCG1. Consequently, at the present time, we cannot account for the differences in localization of ABCG1 in the current study and the study of Gu et al.14

We also performed immunocytochemistry studies to elucidate the localization of endogenous ABCG1. The finding that one anti-ABCG1 antisera lot had enhanced specificity allowed us to demonstrate that endogenous ABCG1 protein is localized to intracellular sites and is undetectable at the cell surface (Figure 2), consistent with our previous biotinylation studies in primary mouse macrophages.11 This conclusion is also consistent with an earlier report from Sturek et al13 who showed that the bulk of ABCG1 (≥90%) was localized to intracellular insulin-containing endocytic vesicles in pancreatic beta cells. Our current studies cannot rule out the possibility that small amounts of ABCG1 can shuttle between endosomes and the plasma membrane under some circumstances. However, our data are consistent with a change in cellular localization of ABCG1 after activation of LXR to induce ABCG1 expression (Figure 2A).

The precise function and substrate of ABCG1 are still largely unknown, yet there is a large body of evidence supporting an intracellular role for this protein. Based on our extensive experiments, we think that the present study confirms that ABCG1 is localized to intracellular vesicles, where it functions to regulate intracellular sterol/lipid homeostasis.

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Disclosures

None.

References


**Highlights**

- The subcellular localization of ATP-binding cassette transporter G1 has been controversial for over a decade.
- Our findings demonstrate that the substitution of leucine to proline at position 550 in mouse ATP-binding cassette transporter G1 is not critical for cellular localization.
- Importantly, our findings confirm previous reports that both endogenous macrophage and ectopically expressed mouse ATP-binding cassette transporter G1 are intracellular and localize to vesicles of the endosomal pathway.
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Online Data Supplement

Supplemental Figure I. mABCG1-L550 and mABCG1-P550 are intracellular and localize to endosomal vesicles. (A-C) Cos-7 cells were transfected with either a control (empty) plasmid or a plasmid expressing untagged ABCG1 (A) or cotransfected with dsRed-Rab5 or dsRed-Rab11 (B-C). Immunofluorescence was determined after incubation of cells with antibody to ABCG1 (Novus Biologicals, Lot E2). Images are at 63X magnification.
Supplemental Figure II. mABCG1-L550-FLAG and mABCG1-P550-FLAG are intracellular and localize to endosomal vesicles. Cos-7 cells were transfected with either a control (empty) plasmid or a plasmid expressing untagged ABCG1 or cotransfected with NPC-1-YFP. Immunofluorescence was determined after incubation of cells with antibody to FLAG (Sigma). Images are at 63X magnification.
Supplemental Figure III. mABCG1-L550-FLAG and mABCG1-P550-FLAG are intracellular and do not localize to the plasma membrane. HEK293 cells were transfected with either a control (empty) plasmid or a plasmid expressing ABCG1-FLAG. Immunofluorescence was determined after incubation of cells with antibodies to FLAG (Sigma) and Na⁺/K⁺-ATPase (Santa Cruz). Images are at 63X magnification. ABCG1-FLAG intracellular localization is indicated by green arrows. Plasma membrane is indicated by positive Na⁺/K⁺-ATPase staining and red arrows.
Online Materials and Methods

Animals
All animals were bred and maintained at the University of California Los Angeles in temperature-controlled, pathogen-free conditions under a 12-h light/dark cycle. The Institutional Animal Care and Research Advisory Committee at the University of California Los Angeles approved all experimental protocols. Abcg1^−/− LacZ knock-in mice (Deltagen)^1 were backcrossed >10 times onto a C57BL/6J background. Control C57BL/6J mice (originally purchased from The Jackson Laboratory) were generated from Abcg1^+/− breeding. All mice were maintained on a chow diet.

Cell Transfection and Immunocytochemistry
Cells were transfected as previously described. Briefly, CHO-K1 and Cos-7 cells were plated on coverslips 1 d prior to transfection and cultured to 80% confluence. Cells were transfected using FuGeneHD (Promega) according to manufacturer’s instructions. Each well was transfected with 200ng of either control empty pcDNA3.1 or pcDNA3.1 expressing L550 or P550 untagged ABCG1 or ABCG1 with three tandem COOH-terminal FLAG epitope tags (3xFLAG) or co-transfected with eYFP-NPC1, dsRed-Rab5 or dsRed-Rab11.

Cells were cultured for 48 h, washed three times with PBS, and fixed in 4% paraformaldehyde for 15 min at 4°C. Immunocytochemistry was performed as previously described. Briefly, cells were treated for 10 min with 0.18% Triton-X100 in PBS, followed by 10 min in 50 mM NH₄Cl. Coverslips were subsequently treated for 30 min in blocking buffer (5% normal goat serum, 1% BSA in TBS), before the cells were costained for 1 h at room temperature with antibodies to ABCG1 (Novus Biologicals; Rabbit polyclonal Lot E2), FLAG (Sigma) or Na⁺/K⁺-ATPase (Santa Cruz) diluted 1:500 in blocking buffer. Cells/coverslips were washed three times in PBS before the addition of either anti-mouse Alexa-fluor 488, anti-mouse Alexa-fluor 546, anti-rabbit Alex-fluor 488, or anti-rabbit Alexa-fluor 546 conjugated secondary antibodies (Molecular Probes). After 30 min at room temperature, cells were washed three more times in PBS and mounted onto slides (Fisher; Superfrost Plus glass slides) using Prolong Gold anti-fade reagent (Molecular Probes). Relative fluorescence was determined using ImageJ software (NIH).

Cholesterol Efflux
Cholesterol efflux experiments from CHO-K1 and Cos-7 cells were performed as previously described. Briefly, cells in quadruplicate were incubated in medium supplemented with 0.2% BSA containing [³H]-cholesterol (1 μCi/mL) for 24 h. The cells were washed extensively prior to addition of HDL-containing media and efflux of [³H]-cholesterol was determined over a 4 h period. Percentage efflux was determined by dividing the [³H]-cholesterol content of the medium by the [³H]-cholesterol content of the medium plus cells.

Isolation of peritoneal macrophages
Mouse primary resident macrophages were isolated from peritoneal cavities in 8 mL serum-free Dulbecco’s Modified Eagles Medium (DMEM). Cells were cultured in 24-well dishes in the presence of 10% fetal bovine serum (FBS), L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin sulfate for 16 h overnight. Cells were treated in the presence or absence of 1 μM LXR agonist GW3965 for 24 h before being fixed in 4% paraformaldehyde.

Luciferase reporter assay
CHO-K1 cells were plated and transfected as previously described. Briefly, cells were transfected with 100 ng pSynSRE plus 5 ng each of the ABCG1 expression plasmid (as
indicated), and 50 ng β-galactosidase expression plasmid as a control to normalize for minor changes in transfection efficiency. 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 manufacturer’s instructions. The luciferase activity was measured using a Centro XS LB 960 luminometer (Berthold Technologies).

**Western blot analysis**

For primary resident peritoneal macrophage studies, cells were harvested in 1X RIPA buffer (Boston Bioproducts) plus protease inhibitor cocktail (Roche Complete Protease Inhibitor Mini EDTA-free tablet) and 25 µg/mL ALLN calpain inhibitor. Total protein lysate (20 µg) was loaded on each lane and separated by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane (Millipore). Primary antibodies were diluted 1:1000 (ABCG1) or 1:5000 (β-ACTIN) in 1x TBS containing 0.1% Tween-20 and 5% non-fat milk. Immune complexes were detected with anti-rabbit (ABCG1) or anti-mouse (β-ACTIN) HRP-conjugated secondary antibodies diluted 1:5000.

**Statistical Analysis**

Statistical analysis was performed using GraphPad Prism 6. For luciferase reporter assays and cholesterol efflux assays, statistical analysis was performed by one-way ANOVA comparing ABCG1 (L550 or P550) to control transfected cells.

**References**

Lipid-poor Acceptors (HPCD > PL > LDL > HDL)

Endoplasmic Reticulum (5% CHOL)

Plasma Membrane

Early Sorting Endosomes

Recycling Endosomes

Late Endosomes/Lysosomes