Title

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Intracellular Localization of Endogenous Mouse ABCG1 Is Mimicked by both ABCG1-L550 and ABCG1-P550

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Abstract

Objective—In a recent article in *Arteriosclerosis, Thrombosis and Vascular Biology*, it was reported that ABCG1 containing leucine at position 550 (ABCG1-L550) was localized to the plasma membrane, whilst ABCG1-P550 (proline at position 550) was intracellular. As the published data on the sub-cellular 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) with untagged and/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 HDL, and in inducing the activity of SREBP-2, presumably as a result of redistributing intracellular sterols away from the endoplasmic reticulum. Importantly, we treated non-transfected primary peritoneal macrophages with an LXR agonist and demonstrate, using immunofluorescence, that while *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.

Keywords

ABC Transporter; cholesterol homeostasis; lipids

Introduction

Although the physiologic 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,

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Disclosures

None.

B-cells, pancreatic beta cells, neurons, and pulmonary epithelial cells.[1–3, 5, 7–10] 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 HDL.[2, 6, 9, 11, 12] It has also been reported that ABCG1 may transport specific phospholipids out of cells.[13]

In a recent paper, Gu *et al.*[14] 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 while transfected mouse ABCG1 (mABCG1-L550) or human ABCG1 (hABCG1-L562) localized to the plasma membrane of HEK293 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]

Since the sub-cellular 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 mouse ABCG1 (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 two fold: to review and utilize our comprehensive catalog of constructs of mouse ABCG1 (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 (Fig. 1A and Supplemental Figure I A). No signal was observed in cells transfected with an empty plasmid (Fig. 1A). In addition, mABCG1-L550 and mABCG1-P550 both co-localize with NPC-1 (Fig. 1B), a marker of late endosomes, and with Rab5 and Rab11, markers for early and recycling endosomes, respectively (Supplemental Figure I B–C). 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 (Fig. 1C) or CHO-K1 cells (Fig. 1D) to exogenous HDL. Parallel immunocytochemistry studies performed following 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 (Supplemental Figure II). In addition, co-localization studies in HEK293 cells transfected with either ABCG1-L550-FLAG or ABCG1-P550-FLAG, demonstrated that ABCG1 localizes to intracellular vesicles and did not co-localize with the Na^+/K^+-ATPase protein, a plasma membrane marker (Supplemental Figure III).

We previously described a sterol-sensitive luciferase reporter assay in which the luciferase activity was dependent on the processing of endogenous SREBP-2.[11] This assay is more robust and sensitive than the cholesterol efflux assay and is sensitive to the level of sterols in the endoplasmic reticulum that control SREBP-2 maturation.[11] We now demonstrate that overexpression of either mABCG1-L550 or mABCG1-P550 resulted in an approximate 4-fold increase in luciferase activity (Fig. 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 HDL or activating SREBP-2 processing. In contrast to the studies of Gu *et al.*,[14] 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 wildtype and *Abcg1*$^{-/-}$ littermate mice. The cells were plated on cover slips and incubated under identical conditions (media containing 10% fetal calf serum) for 24 h and then treated for an additional 24 h with vehicle or the 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 (Fig. 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 (Fig. 2B). Importantly, there was no change in cellular localization in response to treatment with the LXR agonist (Fig. 2A, upper panels). In contrast, there was no change in the low signal derived from *Abcg1*$^{-/-}$ cells (Fig. 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 macrophages approximately 3-fold, and was easily detected with ABCG1 antisera is shown in Fig. 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 in Fig. 2A and C. No signal for ABCG1 protein (~72kDa) was observed in *Abcg1*$^{-/-}$ cells, consistent with high specificity of the antisera for ABCG1 (Fig. 2C). These data are consistent with the proposal

that while *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 utilizing many cell types,[1, 4, 9, 11, 15] we unequivocally show, using multiple cell lines, that ABCG1 localizes to intracellular endosomal vesicles, while it is undetectable at the plasma membrane regardless of whether ABCG1 contains L550 or P550 (Fig. 1A–B, Supplemental Figs. I–III). We also show, using two 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 HDL (Fig. 1C–D). 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, whilst 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 fails to identify the ~72 kDa protein in cells lacking ABCG1 (Fig. 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 wildtype and *Abcg1*$^{-/-}$ cells (Fig. 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 (Fig. 2), consistent with our previous biotinylation studies in primary mouse macrophages.[11] This conclusion is also consistent with an earlier report from Sturek *et al.*[3] 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 inconsistent with a change in cellular localization of ABCG1 following activation of LXR to induce ABCG1 expression (Fig. 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 believe that the present study confirms that ABCG1 is localized to intracellular vesicles, where it functions to regulate intracellular sterol/lipid homeostasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Non-standard Abbreviations

ABCG1	ATP Binding Cassette Transporter G1
HDL	High-density lipoprotein
LXR	Liver X Receptor
SREBP-2	Sterol Regulatory Element Binding Protein 2

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Highlights

- The subcellular localization of ATP Binding Cassette Transporter G1 (ABCG1) has been controversial for over a decade.
- Our findings demonstrate that the substitution of leucine to proline at position 550 in mouse ABCG1 is not critical for cellular localization.
- Importantly, our findings confirm previous reports that both endogenous macrophage and ectopically expressed mouse ABCG1 are intracellular, and localize to vesicles of the endosomal pathway.

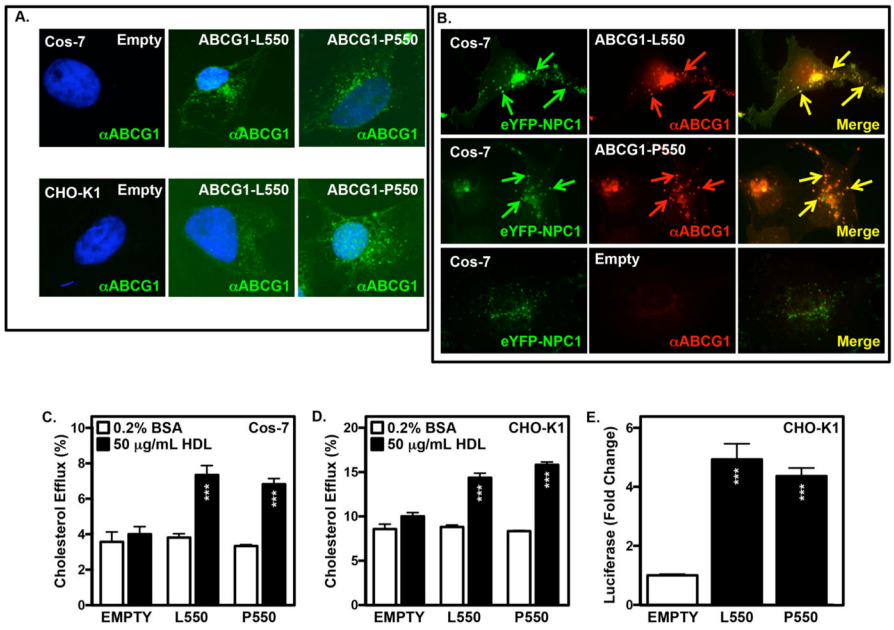

Figure 1. mABCG1-L550 and mABCG1-P550 are intracellular and localize to endosomal vesicles

Cos-7 **(A–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-YFP **(B)**. Immunofluorescence was determined after incubation of cells with antibody to ABCG1 (Novus Biologicals, Lot E2). Images are at 63X magnification. Representative sites of colocalization are indicated by yellow arrows in the merged images **(B, right panels). (C–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 [^{3}H]-cholesterol to 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 h 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 three separate experiments, each performed in quadruplicate **(C–D)** or sextuplet **(E)**. *** $p < 0.001$.

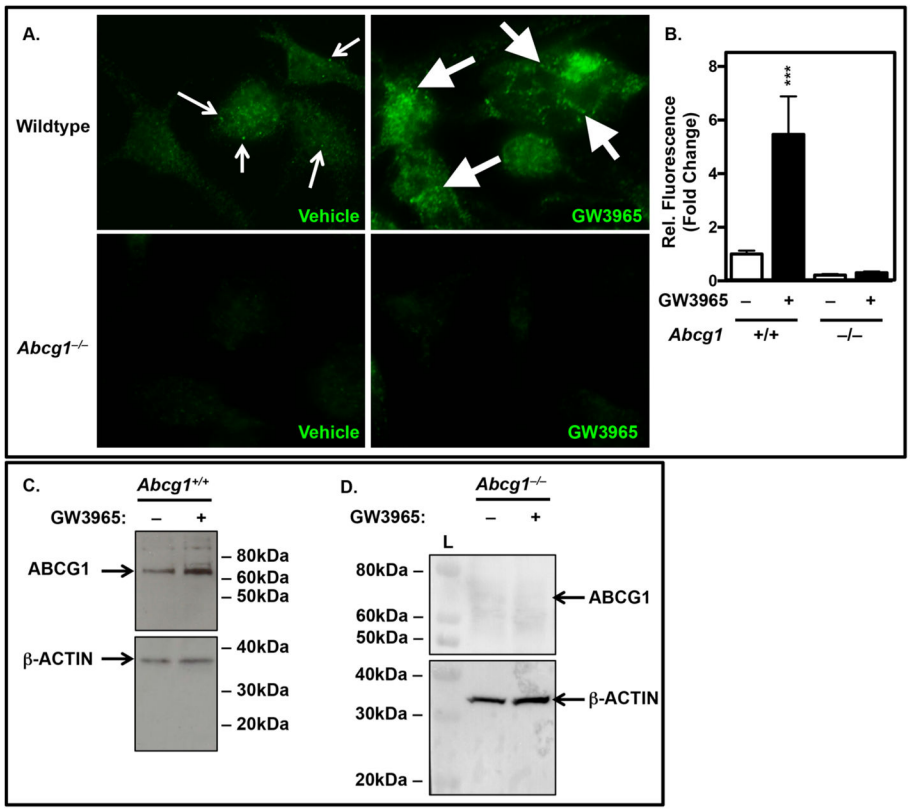

Figure 2. Endogenous mouse ABCG1 is intracellular

(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 h, cells were treated with or without LXR agonist GW3965 (1 μM) for 24 h before either fixing in 4% paraformaldehyde (**A**) as previously described[9, 11], or being harvested in 1X RIPA buffer containing protease inhibitor cocktail and ALLN calpain inhibitor (25 μg/mL) **(C–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 4–7 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–D)** Western blot analysis of wildtype **(C)** and *Abcg1*$^{-/-}$ **(D)** resident peritoneal macrophages (total cell lysates), treated as in **(A)**.