Pim-1L Protects Cell Surface–Resident ABxAC from Lysosomal Degradation in Hepatocytes and Thereby Regulates Plasma High-Density Lipoprotein Level

Akira Katsube,* Hisamitsu Hayashi,* Hiroyuki Kusuhara

Objective—ATP-binding cassette transporter A1 (ABxCA) exerts an atheroprotective action through the biogenesis of high-density lipoprotein in hepatocytes and prevents the formation of foam cells from macrophages. Controlling ABxCA is a rational approach to improving atherosclerotic cardiovascular disease. Although much is known about the regulatory mechanism of ABxCA synthesis, the molecular mechanism underpinning its degradation remains to be clearly described.

Approach and Results—ABxCA possesses potential sites of phosphorylation by serine/threonine-protein kinase Pim-1 (Pim-1). Pim-1 depletion decreased the expression of cell surface–resident ABxCA (csABxCA) and apolipoprotein A-I–mediated [\(^3\)H]cholesterol efflux in the human hepatoma cell line HepG2, but not in peritoneal macrophages from mice. In vitro kinase assay, immunoprecipitation, and immunocytochemistry suggested phosphorylation of csABxCA by the long form of Pim-1 (Pim-IL). Cell surface biotinylation indicated that Pim-IL inhibited lysosomal degradation of csABxCA involving the liver X receptor β, which interacts with csABxCA and thereby protects it from ubiquitination and subsequent lysosomal degradation. Cell surface coimmunoprecipitation with COS-1 cells expressing extracellularly hemagglutinin-tagged ABxCA showed that Pim-IL–mediated phosphorylation of csABxCA facilitated the interaction between csABxCA and liver X receptor β and thereby stabilized the csABxCA–Pim-1L complex. Mice deficient in Pim-1 kinase activity showed lower expression of ABxCA in liver plasma membranes and lower plasma high-density lipoprotein levels than control mice.

Conclusions—Pim-1L protects hepatic csABxCA from lysosomal degradation by facilitating the physical interaction between csABxCA and liver X receptor β and subsequent stabilization of the csABxCA–Pim-1L complex and thereby regulates the circulating level of high-density lipoprotein. Our findings may aid the development of high-density lipoprotein–targeted therapy. (Arterioscler Thromb Vasc Biol. 2016;36:2304–2314. DOI: 10.1161/ATVBAHA.116.308472.)

Key Words: atherosclerosis ■ ATP-binding cassette transporters ■ hepatocytes ■ high-density lipoprotein ■ liver X receptor β ■ Pim-1 kinase

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mechanism underlying the degradation of ABCA1 remains elusive. Although in vitro and animal studies using inhibitors of the protease calpain and proteasome suggest that they cleave and degrade ABCA1,\textsuperscript{1,15} the molecular entity of calpain and the molecular mechanism of the degradation of ABCA1 involving proteasome have not yet been clearly described. On the basis of the report by Hozoji et al\textsuperscript{20} that LXRβ interacts with and post-translationally modulates ABCA1, we recently found that LXRβ protects the cell surface–resident ABCA1 (csABCA1) from ubiquitination through its physical interaction with csABCA1 and not through its transcriptional activity, which prevents lysosomal degradation of csABCA1 through the endosomal sorting complex required for transport (ESCRT) system.\textsuperscript{21,22} This mechanism mediates ABCA1 degradation independently of the pathway involving calpain and proteasome. To gain further insight into this finding, the current study focuses on phosphorylation because phosphorylation can be used to modulate the nature and the strength of protein–protein interactions, thereby regulating protein binding.\textsuperscript{23,24}

ABCA1 has deduced consensus phosphorylation sites (-K/R–K/R–R–K/R–X–S/T-) in the cytosolic region for serine/threonine-protein kinase Pim-1 (Pim-1) encoded by PIM1, a proto-oncogene that is highlighted in research on multiple human malignancies.\textsuperscript{25,26} The activity of Pim-1 is correlated with its cellular expression because it is constitutively active kinase.\textsuperscript{27} Pim-1 is produced as short and long isoforms (Pim-1S, 33 kDa; Pim-1L, 44 kDa) by alternative translation initiation sites,\textsuperscript{28} both of which exhibit comparable in vitro kinase activity.\textsuperscript{29} The 2 isoforms of Pim-1 differ in cellular localization in that Pim-1S is present in the cytosol and nucleus, whereas Pim-1L is primarily localized to the plasma membrane.\textsuperscript{29–31} Therefore, Pim-1S and Pim-1L are considered to regulate distinct substrates. Although the cellular function of Pim-1L has been much less extensively studied than that of Pim-1S, Pim-1L is suggested to form a complex with other cellular components.\textsuperscript{28} Indeed, Pim-1L phosphorylates ABCG2 (an ABC transporter that mediates the efflux of various drugs) and increases its expression; it also confers drug resistance to a prostate cancer cell line.\textsuperscript{33} This prompts us to examine the implication of Pim-1L in the modulation of csABCA1.

In the present study, we show by using the human hepatoma cell line HepG2, mouse macrophages, and Pim-1 kinase activity–deficient mice that Pim-1L interacts and phosphorylates csABCA1, which facilitates the interaction between csABCA1 and LXRβ, retards degradation of csABCA1, and consequently regulates the abundance of csABCA1 and circulating HDL. This mechanism could underpin the hepatocyte-specific response of csABCA1 because Pim-1L is expressed at a much higher level in hepatocytes than in macrophages.

Materials and Methods

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

Results

Decreased Expression and Function of ABCA1 by Pim-1 Depletion in HepG2 Cells but not in Mouse Peritoneal Macrophages

The influence of Pim-1 on the expression and function of ABCA1 was explored in human hepatoma HepG2 cells and mouse peritoneal macrophages (MPMs), both of which express ABCA1 endogenously. Transfection with Pim-1 short interfering RNA (siRNA) markedly suppressed signal intensity of 3 bands that could be detected in HepG2 cells by antibody against Pim-1 (Figure IA) and of 2 of the 3 bands in MPM (Figure IA in the online-only Data Supplement), indicating that all 3 bands in HepG2 cells and the lower 2 bands in MPM represent Pim-1. Under this condition, the expression of ABCA1 in the cell lysates and surface fractions was decreased significantly in HepG2 cells, but not in MPMs. In HepG2 cells, Pim-1 depletion decreased the abundance of ABCA1 by 65% in the cell lysates and 80% in the cell surface fraction. Pim-1 depletion decreased apolipoprotein A-I–mediated [\( ^{3}H \)]cholesterol efflux from HepG2 cells (Figure 1B), which reflects the function of ABCA1.\textsuperscript{1,4,32} Apolipoprotein A-I–mediated [\( ^{3}H \)]cholesterol efflux was 64% and 55% lower in Pim-1 siRNA-transfected HepG2 cells than in control siRNA-transfected HepG2 cells under normal and LXR agonist–treated condition, respectively. No significant difference in apolipoprotein A-I–mediated [\( ^{3}H \)]cholesterol efflux from MPMs and bone marrow–derived macrophages (M0, M1, and M2) was detectable between control (Pim-1\textsuperscript{+/+}) and Pim-1 kinase activity–deficient (Pim-1\textsuperscript{−/−}) mice (Figure IB and ID in the online-only Data Supplement). mRNA expression of ABCA1 was unaffected by Pim-1 depletion in HepG2 cells (Figure 1C).

Presence of Pim-1L in Liver, but not in MPMs, and Decreased Expression of PIM1 mRNA

Under Cholesterol-Depleted Conditions

To gain insight into the regulation of csABCA1 by Pim-1 under physiological and pathophysiological conditions, the expression of Pim-1L, the long isoform of Pim-1 that is tethered in the plasma membrane\textsuperscript{28,31} and could affect csABCA1, was examined in livers and MPMs. The impact of the cellular cholesterol level on the amount of PIM1 mRNA was also evaluated in HepG2 cells.

In livers, Pim-1 was predominantly detected around 50 kDa, which is consistent with the reported molecular weight of Pim-1L,\textsuperscript{28} and was enriched in liver plasma membranes (LPMs; Figure 2A). By contrast, the membrane-tethering
form of Pim-1 was not observed in MPMs and enterocytes (Figure 2A through 2C). The band detected between 50 and 37 kDa in both cells was not derived from Pim-1 because it was not concentrated in the membrane fractions and was unaffected by transfection with Pim-1 siRNA (Figure IA in the online-only Data Supplement). These findings indicate that Pim-1L is highly expressed in liver compared with MPMs and enterocytes. Treatment with lovastatin, a cholesterol-lowering agent, markedly decreased expression of PIM1 mRNA and ABCA1 mRNA, whereas T090137, LXR agonist, had no significant influence on the expression of PIM1 mRNA (Figure 2D).

Phosphorylation of ABCA1 by Pim-1 Through Direct Interaction Between Both Proteins

To examine whether ABCA1 is a substrate of Pim-1, immunoprecipitates with anti-FLAG antibody from HEK293T cells transfected with pcDNA3.1(+)–3×FLAG–ABCA1 or the corresponding empty vector were subjected to in vitro kinase assay, where the specimens were incubated in the presence of [γ-32P]ATP with or without glutathione S-transferase–Pim-1S, which exhibits comparable in vitro kinase activity to Pim-1L.28 Analysis of the products by SDS–PAGE followed by autoradiography showed that glutathione S-transferase–Pim-1S produced a single band above 250 kDa, which is the same molecular weight as ABCA1 (Figure 1A), in the immunoprecipitates from HEK293T cells with ectopic expression of 3×FLAG–ABCA1, but not those from mock-transfected cells (Figure 3A). The band disappeared by the addition of Pim-1 inhibitor to the in vitro kinase assay reaction mixture. The interaction between ABCA1 and Pim-1 was confirmed by the presence of HA–Pim-1L in immunoprecipitates from HEK293T cells with ectopic expression of 3×FLAG–ABCA1 and HA–Pim-1L, but not those from mock-transfected cells (Figure 3B). Consistent with the previous reports,29–31 in immunocytochemistry, HA–Pim-1L was detected not only in cytoplasm but also just beneath

![Figure 1](http://atvb.ahajournals.org/)
the plasma membrane where 3×FLAG–ABCA1 was expressed (Figure II in the online-only Data Supplement). All of these results suggested that Pim-1L phosphorylated ABCA1 through its direct interaction with ABCA1 at the plasma membrane.

**Inhibition of Degradation of csABCA1 by Pim-1L Through Promotion of Interaction Between csABCA1 and LXRβ**

Because Pim-1 had a more dominant effect on the abundance of ABCA1 in the cell surface fractions than that in the cell lysates (Figure 1A), its influence on the degradation of csABCA1 was investigated. The degradation of csABCA1 was markedly facilitated by depletion of Pim-1, but not of ABCA1, in HepG2 cells (Figure 4A; Figure III in the online-only Data Supplement), indicating that Pim-1 itself was implicated in the degradation of csABCA1. The amount of biotin-labeled ABCA1 remaining 4 and 8 hours after incubation was 25% and 2%, respectively, in the Pim-1–depleted HepG2 cells compared with 67% and 46% in the control cells. The accelerated degradation of csABCA1 was abrogated by ectopic expression of HA–Pim-1L (Figure 4B). Treatment with bafilomycin, an inhibitor of lysosomal degradation, eliminated the effect of the depletion of Pim-1 (Figure 4C).

We previously reported that LXRβ reduces the susceptibility of csABCA1 to ubiquitination by its physical interaction with csABCA1 and thereby inhibits lysosomal degradation of csABCA1 through the ESCRT system. To explore the implication of Pim-1 in this machinery, the influence of Pim-1 depletion on the degradation of csABCA1 was determined after treatment with the LXR agonist GW3965, which facilitates the dissociation of LXRβ from csABCA1 and consequently its lysosomal degradation. Pim-1 depletion accelerated the degradation of csABCA1 in HepG2 cells under normal culture conditions but not under GW3965-treated conditions (Figure 5A). Together with the observation that the amount of LXRβ in membrane fraction of HepG2 cells were decreased by 50% by Pim-1 depletion (Figure 5B), this result suggested that Pim-1 modulated the lysosomal degradation of csABCA1 co-operatively with LXRβ. It was corroborated by experiments using COS-1 cells with the ectopic expression of 207HA–ABCA1 (which has an extracellular HA epitope tag between G207 and D208), HA–Pim-1L, and FLAG–LXRβ. In COS-1 cells expressing 207HA–ABCA1, HA–Pim-1L had no impact on the degradation of csABCA1 in the absence of FLAG–LXRβ, but the degradation was delayed in its presence (Figure 5C). Furthermore, Western blot analysis of products from cell surface coimmunoprecipitation, in which only cs207HA–ABCA1 was precipitated because COS-1 cells expressing 207HA–ABCA1 had no impact on the degradation of csABCA1 in the absence of FLAG–LXRβ, showed that HA–Pim-1L enhanced the interaction between 207HA–ABCA1 and FLAG–LXRβ on the plasma membrane (Figure 5D). HA–Pim-1L expression induced 4.5-fold increase in the band intensity of FLAG–LXRβ in the immunoprecipitates. Consistent with the immunocytochemical findings (Figure 3C), the interaction of csABCA1 and Pim-1L was confirmed (Figure 5D). The kinase-inactive Pim-1L mutant (HA-DN-Pim-1L), which enabled interaction with 207HA–ABCA1 to a similar extent as HA–Pim-1L, had much less impact on the interaction between 207HA–ABCA1 and FLAG–LXRβ on the plasma membrane.
To demonstrate the in vivo relevance of the findings obtained in Plasma of Pim-1−/− Mice Decreased ABCA1 Expression in LPMs

interaction between 207HA–ABCA1 and HA–Pim-1L on the

Figure 3. Phosphorylation of ATP-binding cassette trans-

porter A1 (ABCA1) by long form of Pim-1 (Pim-1L) through

their direct interaction. A, Phosphorylation of ABCA1 by Pim-1

in vitro. HEK293T cells were transfected with pcDNA3.1(+) or

pcDNA3.1(+)-3×FLAG–ABCA1. At 48 h after the transfection,

the cells were lysed and immunoprecipitated with M2 beads. The

immunoprecipitates were subjected to in vitro phosphory-

lation by glutathione S-transferase–Pim-1S in the presence of

γ32p. The immunoprecipitates were subjected to autoradiography. A representative result of 3 independent experiments is

shown.

Decreased ABCA1 Expression in LPMs and Decreased HDL Concentration in Plasma of Pim-1−/− Mice

To demonstrate the in vivo relevance of the findings obtained from cells, Pim-1−/− mice, which are deficient in Pim-1 kinase activity, were used. In the primary hepatocytes, the abundance of ABCA1 in the cell surface fraction was 50% lower in Pim-1−/− mice than in Pim-1+/+ mice. No change in its expression between both mice was observed in the cell lysate (Figure 6A). The reduced expression of ABCA1 in the cell lysates of Pim-1-depleted HepG2 cells, but not of the primary hepatocytes from Pim-1−/− mice, could be explained by the fact that ABCA1 expression is relatively concentrated in the cell surface in HepG2 cells compared with its expression in primary hepatocytes. The ratio of ABCA1 in the cell lysate to ABCA1 in the cell surface fraction is 4-fold higher in HepG2 cells than that in the primary hepatocytes (Figure 5 in the online-only Data Supplement). Whereas expression of ABCA1 and LXRβ were not different between the liver homogenates from Pim-1−/− and Pim-1+/+ mice, it was 52% and 75% lower in the LPMs of Pim-1−/− mice compared with the LPMs of Pim-1+/+ mice (Figure 6B). This result supports regulatory mechanism of csABCA1 by Pim-1L and LXRβ in hepatocytes, which are drawn from experiments using COS-1 cells with exogenous expression of these proteins (Figure 5C through 5E). Consistent with finding no change in ABCA1 expression between Pim-1−/− mice and Pim-1+/+ mice in the cell lysate of the primary hepatocytes and the liver homogenates (Figure 6A and 6B), both mice showed similar expression levels of ABCA1 mRNA in liver (Figure 6D). In membrane fractions from enterocytes, ABCA1 expression was increased 1.5-fold in Pim-1−/− mice compared with Pim-1+/+ mice (Figure 6C).

Hepatic ABCA1 predominantly contributes to the biogenesis of HDL particles and maintains the plasma HDL level.6 The plasma HDL concentrations in Pim-1−/− mice were ≈23% lower than those in Pim-1+/+ mice (Figure 6E). Although the influence of Pim-1 deficiency on the plasma HDL level was smaller than that on the abundance of ABCA1, it could be explained by the increase in ABCA1 expression in membrane fractions from enterocytes of Pim-1−/− mice (Figure 6C) because intestinal ABCA1 contributes to the formation of 30% of the plasma HDL pool in the steady state.5

Discussion

In contrast to the growing body of research elucidating the mechanism of ABCA1 synthesis, much less is known about the molecular mechanism underlying its degradation. We previously reported that csABCA1 physically interacts with LXRβ and is thereby protected from ubiquitination and subsequent lysosomal degradation through the ESCRT system.21,22 This regulatory mechanism is independent of the pathway involving calpain and proteasome.22

The current study integrated Pim-1L, a constitutively active serine/threonine-protein kinase, into our previous findings. In vitro studies using HepG2 cells and 32P-labeled ABCA1 expressing COS-1 cells suggested that Pim-1L facilitates the interaction between csABCA1 and LXRβ, resulting in inhibition of lysosomal degradation of csABCA1 through the ESCRT system and an increase in the expression and function of csABCA1 (Figures 1 and 3 through 5). Given that LXRβ has no postulated consensus phosphorylation sites for Pim-1 and increases the association of csABCA1 with Pim-1L (Figure 5E), it is possible that the Pim-1L–mediated phosphorylation of csABCA1 followed by the increased interaction with LXRβ stabilizes the csABCA1–Pim-1L complex itself. Therefore, Pim-1L and LXRβ could mutually facilitate formation of the complex with csABCA1 and efficiently protect csABCA1 from ubiquitination and subsequent lysosomal degradation (Figure 7).

Pim-1L also increases the cell surface expression of ABCG2 and ABCB1 through their phosphorylation.29,31 However, the mechanism responsible differs from that for ABCA1, because Pim-1L–mediated phosphorylation assists
the trafficking of ABCG2 to the plasma membrane by promoting homodimer formation, and that of ABCB1 by inhibiting proteosomal degradation in the endoplasmic reticulum.29,31 At present, how the phosphorylation of csABCA1 by Pim-1L makes it easier for csABCA1 to form a complex with LXRβ remains unclear. It could occur at or near the binding interfaces in csABCA1, which might affect the interaction between both proteins through recognition of a specific phosphorylated serine/threonine by LXRβ and the conferral of binding energy to csABCA1.23,24 Long-range conformational changes through allosteric mechanisms might also be induced by the phosphorylation of a site outside the interacting interfaces in csABCA1.

Consistent with the observations in vitro, Pim-1−/− mice showed a 52% decrease in ABCA1 expression in LPMs and a concomitant 23% decrease in plasma HDL concentrations (Figure 6B and 6E). By contrast, in MPMs and bone marrow–derived macrophages from mice, Pim-1 had no apparent effect on the expression and function of ABCA1 (Figure I in the online-only Data Supplement). The hepatocyte-specific outcome of Pim-1 depletion could be explained by the much lower abundance of membrane-localized Pim-1L in macrophages than that in liver (Figure 2A and 2B). Pim-1 is being studied intensively in the field of multiple human malignancies, including prostate cancers.25,26 This study identified for the first time the physiological function of Pim-1L in HDL homeostasis. The reason Pim-1 is indispensable for controlling the degradation of csABCA1 in hepatocytes, but not in macrophages, may be derived from the physiological function of ABCA1 in these cells. In macrophages, the cholesterol efflux pathway involving ABCA1 and ABCG1 contributes exclusively to the maintenance of cellular cholesterol homeostasis.33,34 By contrast, the cholesterol content in hepatocytes is determined not only by the efflux of cholesterol but also by its synthesis, catabolism to bile acids, and uptake.35 The major role of hepatic ABCA1 is to mediate HDL biogenesis. Given the discrete functions of ABCA1, in macrophages, ABCA1 should be synthesized and degraded immediately to respond to changes in the cellular cholesterol content and thereby protect the macrophages; whereas, in hepatocytes, it is preferable to maintain cellular cholesterol homeostasis by regulating molecules other than ABCA1 involved in the synthesis, catabolism, and uptake of cholesterol, because changes in the circulating HDL level have a physiological impact on many...
Figure 5. Enhancement of interaction between ATP-binding cassette transporter A1 (ABCA1) and liver X receptor β (LXRβ) on the cell surface by long form of Pim-1 (Pim-1L).

A. Effect of Pim-1 on the degradation of cell surface–resident ABCA1 (csABCA1) in LXR agonist–treated cells. Left. HepG2 cells were transfected with control short interfering RNA (siRNA) or Pim-1 siRNA. At 72 h after the transfection, the cells were biotinylated, incubated for 8 h at 37 °C in the absence or presence of LXR agonist (GW3965, 1 μmol/L), and then analyzed as described in Figure 4. Right. Quantification of the amount of the biotin-labeled ABCA1 remaining after the incubation. The band intensity shown on the left was quantified as described in the Materials and Methods and normalized to the intensity at time 0 h in each condition. Each bar represents the mean±SEM of triplicate determinations. **P<0.01; ***P<0.001.

B. Effect of Pim-1 on LXRβ expression in membrane fraction. (Continued)
other tissues and the liver. This hypothesis is corroborated by the finding that the accumulation of cellular cholesterol induces more ABCA1 transcripts involving LXR in peripheral cells, like macrophages, than in hepatocytes,\textsuperscript{36–38} and that the half-life of csABCA1 is longer in HepG2 cells than that in MPMs (8 hours in HepG2 cells and 4 hours in MPMs\textsuperscript{21}). Therefore, it is conceivable that the phosphorylation of csABCA1 by Pim-1L is needed in hepatocytes only to confer...
some degree of resistance to ubiquitination of csABCA1 and its subsequent lysosomal degradation and to maintain circulating HDL as constant as possible.

As expected, because Pim-1 is a target of miR-33, which is present in the intron of SREBP-2 and induced by cholesterol depletion, mRNA expression of Pim-1 was decreased under cholesterol-depleted conditions (Figure 2D). miR-33 controls cholesterol homeostasis by targeting genes implicated in cholesterol transport, including ABCA1. Delivery of miR-33 into mice causes a 22% decrease in the plasma HDL level by reducing the hepatic expression of ABCA1. Therefore, hepatic ABCA1 might be suppressed by miR-33 directly during the synthetic process through destabilization of ABCA1 mRNA and repression of its translation and indirectly during the degradation process by facilitating the lysosomal degradation of csABCA1 by a decrease in Pim-1L expression (Figure 7). By contrast with the depletion of cellular cholesterol, treatment with the LXR agonist had no significant impact on PIM1 level (Figure 2D).

From the viewpoint of cell protection, it seems disadvantageous to facilitate degradation of csABCA1 under conditions of high cellular cholesterol because this adaptation decreases expression of csABCA1 and cholesterol efflux by ABCA1, which induces cell toxicity by abnormal deposition of cellular cholesterol. However, given that the cellular cholesterol level in hepatocytes is modulated by its synthesis, catabolism to bile acids, and uptake, as well as its efflux by ABCA1, and that transcription of ABCA1 mRNA is markedly induced in response to cholesterol accumulation, the machinery identified in the current study might contribute to limiting variation of circulating HDL by acutely increasing hepatic ABCA1 expression.

In conclusion, our current study suggests that Pim-1L interacts with and phosphorylates csABCA1, which facilitates the interaction between csABCA1 and LXRβ and thereby prevents its ubiquitination and subsequent lysosomal degradation through the ESCRT system. Given that the interaction of the phosphorylated csABCA1 by Pim-1L with LXRβ stabilizes the csABCA1–Pim-1L complex, this machinery composes the positive feedback loop to protect csABCA1 efficiently from ubiquitination. Through this molecular mechanism, Pim-1 regulates the hepatic expression of ABCA1 and circulating HDL levels. There is an inverse relationship between plasma HDL concentration and atherosclerotic cardiovascular disease risk. Therefore, there has been great interest in targeting HDL therapeutically. Our findings point to the possibility that modulating the molecular mechanism underlying lysosomal degradation of csABCA1 may be a rational strategy to increase hepatic ABCA1 expression and raise circulating HDL levels. Ubiquitination acts as a signal for the sorting of internalized csABCA1 to lysosomal degradation in the early endosome. The ubiquitination of substrate proteins is mediated by the covalent attachment of ubiquitin via the sequential action of 3 enzymes: a ubiquitin-activating enzyme E1, a ubiquitin-conjugating enzyme E2, and a ubiquitin ligase E3. Among
Figure 7. Proposed model for regulation of cell surface–resident ABCA1 (csABCA1) by long form of Pim-1 (Pim-1L) and liver X receptor β (LXRβ) in hepatocytes. Pim-1L phosphorylates csABCA1, which facilitates interaction between csABCA1 and LXRβ and then stabilizes the csABCA1–Pim-1L complex. Because LXRβ inhibits ubiquitination of csABCA1 through its physical interaction and consequently the lysosomal degradation of csABCA1 involving the endosomal sorting complex required for transport (ESCRT) system,21,22 the machinery composed of Pim-1L, and LXRβ is the positive feedback loop to protect csABCA1 efficiently from ubiquitination and consequently controls the abundance of csABCA1 and high-density lipoprotein (HDL) biogenesis. The expression of Pim-1L alters depending on the cellular cholesterol level. Thus, Pim-1L contributes to the regulation of HDL homeostasis. E3s indicates ubiquitin ligases; and P, phosphorylation.

these 3 enzymes, E3 is considered the enzyme that ensures the correct timing, localization, and specificity of the ubiquitination reaction.41 The E3 ubiquitin ligase inducible degrader of the low-density-lipoprotein receptor (IDOL), which is transcriptionally regulated by cellular cholesterol level through LXR, controls the amount of low-density lipoprotein receptor, and thereby affects cholesterol homeostasis. The abundance of hepatic low-density lipoprotein receptor in Pim-1−/− mice was the same as in Pim-1+/+ mice (Figure 6A). Therefore, it is likely that the ubiquitination of csABCA1 is mediated independently of the LXR–Idol–low-density lipoprotein receptor axis.38 Further studies to identify and characterize the E3 ligases that mediate ubiquitination of csABCA1 should pave the way to modulating the lysosomal degradation of csABCA1 and realizing HDL-targeted therapies.

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Disclosures
None.

References


**Highlights**

- **Pim-1L, a constitutively active serine/threonine-protein kinase, phosphorylated and protected cell surface–resident ABCA1 (csABCA1) from lysosomal degradation in hepatocytes and thereby regulating circulating high-density lipoprotein levels.**

- **Pim-1L-mediated phosphorylation of csABCA1 facilitated interaction between csABCA1 and liver X receptor β, which confers resistance to the ubiquitination of csABCA1 and its subsequent lysosomal degradation involving the endosomal sorting complex required for transport system.**

- **The plasma high-density lipoprotein levels can be modulated by manipulating the lysosomal degradation of hepatic csABCA1.**
Pim-1L Protects Cell Surface–Resident ABCA1 From Lysosomal Degradation in Hepatocytes and Thereby Regulates Plasma High-Density Lipoprotein Level

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Materials and methods
Reagents and Antibodies

Human recombinant apoA-I was purchased from BioDesign (Saco, ME). [3H]cholesterol and [γ-32P]ATP were obtained from PerkinElmer (Boston, MA). Recombinant glutathione S-transferase (GST)-Pim-1S was purchased from Abcam (Cambridge, UK). The following antibodies were used; mouse monoclonal anti-ABCA1 (Abcam), mouse monoclonal anti-Pim-1 (sc13513; Santa Cruz Biotechnology, Dallas, TX), rabbit monoclonal anti-Pim-1 (ab75776; Abcam), mouse monoclonal anti-Na+, K+-ATPase α1 subunit (NaK; Abcam), rabbit polyclonal anti-LXRβ antibody (ab28479; Abcam), rabbit polyclonal anti-FLAG epitope antibody (Sigma-Aldrich, St. Louis, MO), rat monoclonal anti-hemagglutinin (HA) epitope antibody (Roche, Basel, Switzerland), and mouse monoclonal anti-HA epitope antibody (Santa Cruz Biotechnology). Alexa-labeled secondary antibodies were purchased from Molecular Probes (Life Technologies, Carlsbad, CA). All other chemicals were of analytical grade.

siRNA

The sequence of Stealth RNAi against Pim-1 (Pim-1 siRNA, Life Technologies) is as follows: sense 5’-CCAUGGAAGUGGUCCUGCUAGAA-3’ and antisense 5’-UUCUUCAGCAGGACCACUUCAUGG-3’ for human Pim-1; and sense 5’-CCCAUGGAAGUGGUCCUGUAAGA-3’ and antisense 5’-UCUUCAACAGGACCACUUCAUGG-3’ for mouse Pim-1; sense 5’-GACCAAAGUGAUGAUGACCACUUAA-3’ and antisense 5’-UUAAGUGGUCAUCAUCACUUUGGC-3’ for human ABCA1. Stealth RNAi Negative Control High GC Duplex (Life Technologies) was employed as control siRNA.

Plasmid Vector

The pcDNA3.1(+) vector (Life Technologies) containing human ABCA1 cDNA N-terminally tagged with 3×FLAG (pcDNA3.1(+)–3×FLAG–ABCA1) and human ABCA1 with an influenza HA epitope between G207 and D208 (pcDNA3.1(+)–207HA–ABCA1) were constructed as previously described.1-3 The pCMV vector containing human LXRβ cDNA N-terminally tagged with FLAG (pCMV–FLAG–LXRβ) and the pOTB7 vector containing human Pim-1L cDNA were provided by RIKEN BRC (Ibaraki, Japan). The Pim-1L cDNA was tagged N-terminally with an influenza HA epitope and subcloned into pcDNA3.1(+) (pcDNA3.1(+)–207HA–ABCA1).
HA–Pim-1L). To generate the kinase-inactive Pim-1 mutant (pcDNA3.1(+)–HA–DN-Pim-1L), the lysine residue at position 158 of Pim-1L was mutated to methionine using the site directed mutagenesis as described previously ⁴.

Cells

HepG2, HEK293T, and COS-1 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies) containing 10% fetal bovine serum (10% FBS/DMEM) in the presence (HepG2) or absence (HEK293T and COS-1) of nonessential amino acids (Life Technologies). All cells were cultured at 37°C under an atmosphere of 5% CO₂ in air at 95% humidity.

Mice

Pim-1 activity-deficient (Pim-1⁻/⁻) mice that were generated as described⁵ and maintained on a C57/BL6J background were kindly gifted by Dr. Berns (Division of Molecular Genetics, the Netherslands Cancer Institute, the Netherlands). Pim-1⁻/⁻ mice were mated to C57/BL6J mice (CLEA Japan, Tokyo, Japan). The generated heterozygous mice were bred to maintain control (Pim-1⁺/⁻) mice and Pim-1⁻/⁻ mice. All animals were maintained under standard conditions with a reverse dark–light cycle and were treated humanely. Food and water were available ad libitum. The studies reported in this article were conducted in accordance with the guidelines provided by the Institutional Animal Care and Use Committee (Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo, Japan). At 9–14 weeks of age, the male mice were genotyped, fasted for 12 h, and used for the experiments. Measurement of plasma HDL levels was outsourced to Skylight Biotech (Akita, Japan).

Preparation of the primary hepatocytes

Pim-1⁺/⁻ and Pim-1⁻/⁻ mice were fasted for 12 h and anesthetized with pentobarbital sodium. Hepatocytes were isolated using the collagenase perfusion method ⁶–⁷, suspended in Krebs–Henseleit buffer, and subjected to cell surface biotinylation.

Preparation of the Liver Plasma Membrane Fractions (LPMs)

The LPMs were prepared from the livers of mice after they were fasted for 12 h as described previously.² ⁸–¹⁰
Preparation of the Membrane Fractions of Enterocytes

Mice were fasted for 12 h, anesthetized with pentobarbital sodium, and perfused systemically using PBS. The luminal side of the small intestine was washed with cold PBS containing a protease inhibitor cocktail tablet (Roche) (PBS/PI) using an 18 gauge needle and syringe. The mucosa of the top half of the small intestine was gently scraped off with a slide glass and then thoroughly homogenized with cold PBS/PI. The homogenates were centrifuged at 2,080g for 15 min at 4°C. The supernatant was collected carefully and then ultracentrifuged at 80,000g for 60 min at 4°C (50.2 Ti rotor, Beckman, Brea, CA). The pellet was resuspended in PBS/PI and stored at –80°C before use in Western blot analysis.

Preparation of the Membrane and Cytosol Fraction from HepG2 cells

HepG2 cells were cultured on 10-cm dishes and transfected with control or Pim-1 siRNA using Lipofectamine RNAiMax (Life Technologies). At 72h after the transfection, the cells were harvested and disrupted by sonication in isotonic buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 250 mM sucrose) containing a protease inhibitor cocktail. The post-nuclear supernatant were obtained by centrifugation of the specimens at 2,800g for 10 min at 4°C and subsequent at 12,000g for 10 min at 4°C. Membrane fractions in the post-nuclear supernatant were pelleted by ultracentrifugation at 100,000g for 20 min at 4°C and washed once by the isotonic buffer. The resultant supernatants of the ultracentrifugation were collected as the cytosolic fraction. The prepared membrane and cytosolic fractions were stored at –80°C before use in Western blot analysis.

Preparation of Peritoneal Macrophages From Mice

Peritoneal macrophages (MPMs) were isolated from mice that were injected intraperitoneally (2 mL/mouse) with 4.05% autoclaved thioglycolate broth medium (Becton, Dickinson and Company, Sparks, MD) as described previously.2, 3, 11

Preparation of bone marrow-derived macrophages (BMDMs) from mice

Bone marrow cells were isolated from the femurs and tibias of mice and flushed with sterile PBS using a 25 gauge needle and syringe. Then, the obtained cells were incubated with hemolysis buffer (140 mM NH₄Cl, 17 mM Tris-HCl; pH 7.65) for 10 min at room temperature, washed twice with sterile PBS, and differentiated into BMDMs (M0
macrophage) by incubation with DMEM containing the supernatant of L929 cells at a final concentration of 30 % (v/v) (30% L929/DMEM). To polarize M0 macrophages into M1 or M2 macrophages, M0 macrophages were cultured in 30% L929/DMEM with 20 ng/mL mouse IFN-γ (Biolegend, San Diego, CA) for 48 h followed by stimulation with 10 ng/mL LPS (Sigma-Aldrich) for 24 h or cultured in 30% L929/DMEM with 40 ng/mL mouse IL-4 (Biolegend) for 48 h.

**Cell Surface Biotinylation**

HepG2 cells and COS-1 cells were seeded in 12-well plate to examine the expression level of csABCA1 or 6-well plate to measure degradation rate of csABCA1 and transfected with control or Pim-1 siRNA using Lipofectamine RNAiMax and with the indicated vector using XtremeGeneHP (Roche), according to the manufacturer’s instructions. At 72 h (HepG2 cells) or 48 h (COS-1 cells) after the transfection, the cell was biotinylated and lysed. When determining the rate of degradation of the csABCA1, the biotinylated cells were incubated at 37°C for the indicated time period and then lysed as described previously.\(^2\)\(^,\)\(^3\) The primary hepatocytes were biotinylated just after their isolations from Pim-1\(^+/+\) and Pim-1\(^-/-\) mice.

90% of the lysates from the biotinylated cells were precipitated with streptavidin to prepare cell surface fractions.\(^8\),\(^10\) Isolated biotinylated protein was analyzed by Western blot analysis.

**Immunoprecipitation**

HEK293T and COS-1 cells were transfected with the indicated vectors using XtremeGeneHP. After 72 h culture, these cells were lysed in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100; pH 7.5) with a protease inhibitor cocktail (Sigma-Aldrich) and PhosSTOP (Roche) and then incubated with mouse monoclonal anti-FLAG epitope antibody conjugated to affinity gel (M2-beads, Sigma-Aldrich) for 2 h at 4°C. Immune complexes were precipitated, washed five times with 1 mL of lysis buffer, and eluted for 30 min with 3×FLAG peptide (Sigma-Aldrich) at 4°C. The specimens were analyzed by Western blot analysis.

**Cell Surface Immunoprecipitation (csIP)**

COS-1 cells were transfected with pcDNA3.1(+)--\(^{207}\)HA--ABCA1, which contained
the cDNA of the extracellularly HA-tagged ABCA1\(^1,2\), pCMV–FLAG–LXR\(\beta\), pcDNA3.1(+)–HA–Pim-1L, and/or the corresponding empty vector using PEI-MAX (Polysciences, Warrington, PA), according to the manufacturer’s instructions. After 72 h culture, these cells were incubated with PBS containing 5 mM EDTA at 37°C for 20 min. The harvested cells were collected, suspended in PBS containing anti-HA antibody (10 ng/μL) and 5% BSA at 4°C, incubated for 2 h, washed twice with PBS, and then lysed in lysis buffer (20 mM Tris-HCl, 50 mM EDTA, 50 mM ethylene glycol tetraacetic acid, and 1% Triton X-100; pH 7.5) containing protease inhibitor cocktail and PhosSTOP. The specimens were bound to Dynabeads Protein A (Life Technologies), washed three times with lysis buffer, eluted for 1 h at 37°C with PBS containing HA peptide (100 ng/μL, Roche), and subjected to Western blot analysis.

**Western Blot Analysis**

Specimens were loaded into wells of a 7% or 10% SDS-polyacrylamide gel (SDS-PAGE) with a 3.75% stacking gel, electrophoresed, and subjected to Western blot analysis as described previously.\(^2,3\) Immunoreactivity was detected using an ECL Advance Western Blotting Detection Kit (GE Healthcare Life Sciences, Piscataway, NJ) by ImageQuant LAS 4000 (GE Healthcare Life Sciences) at high resolution and auto exposure. The intensity of the band indicating each protein was quantified by Multi Gauge software v. 2.0 (Fujifilm, Tokyo, Japan).


HepG2 cells, mouse peritoneal macrophages (MPMs), and bone marrow-derived macrophages (BMDMs) (M0, M1, and M2) were seeded, cultured for 24 h, and subjected to the assay. ABCA1-mediated cholesterol efflux was measured as described previously.\(^3\)

**Quantitative PCR (qPCR)**

RNA was isolated using ISOGEN (Nippon gene, Tokyo, Japan) according to the manufacturer’s instructions. Reverse transcription was performed using ReverTra Ace\(^®\) qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). mRNA expression level of target genes was determined by real-time qPCR using a LightCycler 480 system II (Roche Diagnostics, Mannheim, Germany), the appropriate software (Ver. 3.53; Roche Diagnostics), and THUNDERBIRD SYBR qPCR Mix (TOYOBO) as described previously.\(^6\)
The following primer sequences were used: 5'- GGACCGAGTGCTACAGTCTCCAG-3' (forward) and 5'-CTCCGAGACCAGTGTAGAG-3' (reverse) for human PIM1, 5' - AGGTGGCCTGCTATTATCTTCC-3' (forward) and 5' - GCCTCCGAGCATCTGAGAAGGC-3' (reverse) for human ABCA1, 5'- CCAGACAGTTGATGGTG-3' (forward) and 5' -CCTGTGGTGAACGGAATTCTT-3' (reverse) for mouse ABCA1, 5'- ACGACCCTTATTAC-3' (forward) and 5'- TCCACGACCATCTCAGC-3' (reverse) for human GAPDH, 5'- AGAATCCCTCCGTATCC-3' (forward) and 5' -CACATGPGGATAGAACAC-3' (reverse) for mouse Gapdh, 5'- CCAGACCCCTTAC-3' (forward) and 5'- CCTGTGTGATGTAATC-3' (reverse) for mouse IL-1β, and 5'-CCAATCCAGCTAATCTCCCT-3' (forward) and 5'-ACCCAGTAGCAGTC-3' (reverse) for mouse Fizz-1. Gene expression for each reaction was normalized by expression of the corresponding species of GAPDH/Gapdh.

**Immunocytochemistry**

COS-1 cells were transfected with the indicated vectors using XtremeGeneHP and seeded onto glass cover slips (Matsunami Glass, Osaka, Japan) in 12-well plates at a density of 1.0 × 10^5 cells per well. After 24 h culture on glass cover slips, the cells were fixed with 4% paraformaldehyde/PBS for 10 min and permeabilized in 0.1% saponin/PBS for 10 min. Nonspecific binding was blocked with 3% BSA/PBS for 30 min, and cells were stained with goat monoclonal anti-FLAG epitope antibody and rat monoclonal anti-HA epitope antibody for 1 h followed by Alexa Fluor 488 donkey anti-rat immunoglobulin G and Alexa Fluor 546 donkey anti-goat immunoglobulin G for 1 h. The staining procedures were performed at room temperature. After mounting onto glass slides with Vectashield medium (Vector Laboratories, Burlingame, CA), the cells were visualized by confocal microscopy using a Leica TCS SP5 II laser scanning confocal microscope (Leica, Solms, Germany).

**Statistical Analysis**

Graphs include means ± standard error of the mean (SEM). Differences between two variables and multiple variables were assessed at the 95% confidence level using Student t tests and analysis of variance with post hoc Dunnett tests, respectively. The data were analyzed using Prism software (GraphPad Software, La Jolla, CA).
References


10. Hayashi H and Sugiyama Y. 4-phenylbutyrate enhances the cell surface expression and the transport capacity of wild-type and mutated bile salt export pumps. *Hepatology*
Supplemental Figure I

A.  

<table>
<thead>
<tr>
<th>siRNA:</th>
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<tr>
<td>Pim-1</td>
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Cell lysate

Cell surface fraction

(kDa)

B.

ApoA-I-mediated efflux (%)

Pim-1+/+  Pim-1/−

C.

IL-1β mRNA (relative to 10^−17)

M0  M1  M2

Pim-1+/+  Pim-1/−

Fizz1 mRNA (relative to 10^−17)

M0  M1  M2

Pim-1+/+  Pim-1/−

D.

Apo A1-mediated efflux (%)

M0  M1  M2

Pim-1+/+  Pim-1/−

Supplemental Figure I----Influence of Pim-1 depletion on expression and function of ABCA1 in macrophages.

(A) ABCA1 expression in the cell lysates and cell surface fractions. MPMs were transfected with control siRNA or Pim-1 siRNA. At 72 h after the transfection, the cells were biotinylated and analyzed by Western blot analysis as described in Figure 1A. #, non-specifie band. (B-D) MPMs (B) and M0, M1, and M2 macrophages (C, D) were prepared from Pim-1+/+ and Pim-1/− male mice as described in Materials and Methods and used for cholesterol efflux assay (B, D), and isolation of RNA and subsequent qPCR (C). (B, D) ApoA-I-mediated cholesterol efflux. MPM (B) and M0, M1, and M2 macrophages (D) were analyzed as described in Figure 1B. (C) mRNA expression of macrophage markers. The expression of IL-1β (M1 marker) and Fizz1 (M2 marker) in each reaction was normalized to that of Gapdh. In (B-D), each bar represents the mean ± SEM of triplicate determinations. In (A-D), a representative result of more than two independent experiments is shown.
Supplemental Figure II

Supplemental Figure II—Cellular localization of ABCA1 and Pim-1L.
COS-1 cells were transfected with pcDNA3.1(+)–3 × FLAG–ABCA1 and pcDNA3.1(+)–HA–Pim-1L. At 48 h after the transfection, the cells were fixed and stained for confocal immunofluorescence microscopy, as described in the Materials and Methods. A representative image of more than 30 cells from three independent experiments is shown. Yellow in the merged images indicates co-localization. Scale bar, 10 µm.
Supplemental Figure III

Supplemental Figure III----No influence of ABCA1 depletion on degradation rate of csABCA1 in HepG2 cells.
HepG2 cells were transfected with control siRNA or ABCA1 siRNA. At 72 h after the transfection, the cells were lysed (A) or biotinylated, incubated for 6 h at 37°C, lysed, and precipitated with streptavidin (B). (A, B Left) The prepared specimens were analyzed by Western blot analysis. A representative result of two independent experiments is shown. In (A), the signal intensity of ABCA1 relative to that of NaK is presented below panel. (B, Right) quantification of the amount of the biotin-labeled ABCA1 remaining after the incubation. The band intensity shown on the left was quantified as described in the Materials and Methods. Each bar represents the mean ± SEM of triplicate determinations.
Supplemental Figure IV-----ABCA1 expression in the cell lysates and cell surface fractions of HepG2 cells and primary hepatocytes. 

*Left*, HepG2 cells and primary hepatocytes from male C57/BL6J mice were biotinylated and lysed. 80% of the cell lysate were precipitated with streptavidin to prepare cell surface fraction. 20% of the cell lysate and the cell surface fraction were analyzed by Western blot analysis. *Right*, the ratio of the amount of ABCA1 in the cell surface fraction to that in the cell lysate. The band intensity shown on the left was quantified as described in the Materials and Methods. Each bar represents the mean ± SEM of three independent experiments. ***, P <0.001