

Lipid outward translocation by ABC proteins

Kohjiro Nagao¹, Yasuhisa Kimura², Michinori Mastuo², and Kazumitsu Ueda^{1,2}

¹*Institute for Integrated Cell-Material Sciences (iCeMS), Kyoto University, Kyoto 606-8502, Japan*

²*Laboratory of Cellular Biochemistry, Division of Applied Life Sciences, Kyoto University Graduate School of Agriculture, Kyoto 606-8502, Japan*

Abstract

In humans, about 50 ABC proteins play physiologically important roles. Many ABC proteins are involved in lipid outward translocation and lipid homeostasis in the body and defects in their functions causes various diseases; however, the precise mechanisms of substrate transport remain unclear. In bacteria, several ABC proteins are involved in the transport of lipoproteins and lipopolysaccharides from the inner to outer membrane, and their functions are prerequisite for their survival. Their functions can be divided into “flip-flop” and “projection”. In this review, human ABC proteins are compared to bacterial proteins to elucidate their mechanisms.

1. Introduction

The lipid bilayer is the ideal barrier for life to maintain concentrations of solutes in cytosol and selectively segregate biochemical reactions from the external environment. Because water-soluble molecules and inorganic ions hardly dissolve in lipid bilayers or diffuse across them, specific membrane proteins, transporters and channels, which transport water-soluble molecules and inorganic ions across lipid bilayers, have evolved and cells have achieved competency to ingest essential nutrients, excrete metabolic waste products, and regulate intracellular ion concentrations. By contrast, because hydrophobic molecules readily dissolve in lipid bilayers and diffuse across them, they have been considered to move according to their concentration gradient across membranes in cells and in the body.

We identified human *MDR1* gene (now called *ABCB1* by the HUGO (Human Gene Organization) Nomenclature Committee) amplified and overexpressed in multidrug-resistant carcinoma cell lines in 1986 [1,2], and showed that it confers multidrug resistance depending on its own expression. Afterwards, *ABCB1* turned out to code for P-glycoprotein [3], a surface glycoprotein overexpressed in drug-resistant Chinese hamster ovary cell mutants [4]. *MDR1* extrudes a very wide array of structurally dissimilar compounds, all lipophilic or amphiphilic, and ranging in mass from approximately 300 to 2000 Da, including cytotoxic drugs that act on different intracellular targets [5-7]. *MDR1* is clinically important because it not only confers multidrug resistance to cancer cells but also affects the pharmacokinetics of various drugs [8,9]. This discovery suggested that lipophilic endogenous compounds, which have been considered to

move according to their concentration gradient in cells and in the body, could be also actively transported across membranes in an ATP-dependent manner. Indeed, it was revealed in 1993 that murine ABCB4 (also called MDR2), highly homologous to MDR1, is involved in phosphatidylcholine (PC) transport and is indispensable for bile formation [10].

Although several mechanisms have been proposed for substrate recognition and transport by MDR1 and other ABC proteins [11-14], they remain to be clarified. Four processes can be predicted for outward lipid transport across membranes, as seeing in Fig. 1. (a) The substrates enter the transporter from the inner leaflet and exit from the transporter into the outer leaflet. Substrates could be moved from one substrate binding site to the other in the transporter in a “flip-flop” way. Substrates may dissolve into the inner leaflet from cytosol, and exit to an exogenous environment from the outer leaflet. (b) Substrates enter the transporter from the inner leaflet as model (a) but exit the transporter directly to an exogenous environment. Acceptors could be required for the substrates to solve in an exogenous environment. Without acceptors, the substrates would readily return to the outer leaflet of the lipid bilayer. (c) Amphiphilic substrates directly enter the transporter from cytosol and exit directly to an exogenous environment. This is similar to the transport mechanisms for ions and solutes. (d) The substrates enter the transporter from the outer leaflet and exit the transporter directly to an exogenous environment. Acceptors would be required for the substrates to solve in an exogenous environment also in this case. The movement of substrates in this model can be called “projection” to the exogenous environment.

In this review, the mechanism of outward transmembrane lipid transport by physiologically important human ABC proteins will be discussed. First, ABC proteins involved in the biogenesis of the outer membrane in Gram-negative bacteria are described. Their transport mechanisms have been studied intensively and are highly suggestive when considering the mechanism of human ABC proteins.

2. Bacterial ABC proteins in lipoprotein transport

The outer membrane is essential for Gram-negative bacteria, such as *Escherichia coli* (*E. coli*). One of the major functions of the outer membrane is a permeability barrier to hydrophobic substances. It is composed of four major components: lipopolysaccharide (LPS), phospholipids, β -barrel proteins, and lipoproteins. *E. coli* possesses at least 80 species of lipoproteins anchored to the periplasmic face of the outer membrane thorough acyl chains attached to their N-terminal Cys residues. Lipoproteins are synthesized as precursors in the cytoplasm, and translocated across the inner membrane through a Sec translocon. The precursor proteins are then sequentially processed to the mature acylated form on the periplasmic face of the inner membrane. Because the signal peptide is processed by a signal peptidase, the mature lipoproteins are anchored via only three acyl chains (Fig. 2a). Extensive biochemical studies by Tokuda's group [15] revealed that the outer membrane protein LolB, the periplasmic protein LolA, and a complex composed of LolC, LolD, and LolE in the inner membrane are involved in lipoprotein transport. This LolCDE complex belongs to the ABC transporter superfamily. LolC and LolE are membrane subunits, each of which spans the membrane four

times, while LolD is a nucleotide binding subunit possessing typical motifs conserved among the ABC transporter superfamily.

The release of the lipoprotein from the inner membrane is presumed to proceed as illustrated in Fig. 2c: the lipoprotein is recognized by LolCDE on the periplasmic surface of the inner membrane. Formation of the LolCDE/lipoprotein complex increases the affinity of LolD for ATP (step 1). ATP binding to LolD causes a conformational change in LolCDE and decreases the strength of the hydrophobic interaction between LolCDE and lipoprotein (step 2). ATP hydrolysis presumably further weakens the hydrophobic interaction of the LolCDE/lipoprotein complex, but lipoproteins remain associated with LolCDE until LolA is added (step 3). The hydrophobic cavity of LolA opens on the binding of lipoproteins (step 4). Release of inorganic phosphate and ADP from LolD leads to the original conformation of LolCDE (step 5). The process of lipoprotein release by LolA and LolCDE does not include transmembrane movement but occurs on one side of the membrane [16,17]. Thus, the outward transport process mediated by LolCDE represents the model (d) in Fig. 1, in which the substrate is “projected” from the outer leaflet of the membrane.

3. Bacterial ABC proteins in LPS transport

LPS is an essential component of the outer membrane in most Gram-negative bacteria as lipoproteins. LPS consists of a hydrophobic membrane anchor, lipid A moiety, linked to a short core oligosaccharide and a distal long chain polysaccharide known as O-antigen. The lipid A-core moiety is synthesized on the cytoplasmic surface of the inner membrane and is then

transported across the inner membrane by an ABC transporter, MsbA. The movement of the lipid A-core moiety mediated by MsbA is “flip-flop” from the inner leaflet to the outer leaflet of the inner membrane [18]. Thus, the outward transport process mediated by MsbA represents the model (a) in Fig. 1. O-antigen is synthesized separately and is present in its completed form at the periplasmic face of the inner membrane, attached to the carrier lipid (undecaprenyl diphosphate). O-antigen repeat units are then polymerized in the periplasm and ligated to the lipid A-core moiety [19].

Recently, several proteins have been identified to be involved in LPS transport from the inner to outer membrane of *E. coli* [20,21]. Two transmembrane proteins, LptF and LptG, form a complex with LptB, a cytosolic ATPase, with a subunit stoichiometry of 1:1:2, just like LolCDE [22]. LPS is predicted to target the outer membrane through a pathway analogous to the lipoprotein targeting system. LptA may play a chaperone role in the transport of LPS across the periplasm (Fig. 2b).

It is intriguing that two types of outward transporter are involved in LPS transport from the inner to outer membrane. One is MsbA, which moves lipid A-core moiety in a “flip-flop” manner as does model (a), and the other is LptBFG, which possibly “projects” mature LPS to LptA, just like LolCDE as in model (d). There may be two possible reasons why two separate systems are required for bacteria to transport LPS: i) the two transport steps are separated because polymerized O-antigen should be ligated to the lipid A-core moiety on the periplasmic face of the inner membrane after being transported by MsbA and then recognized by LptBFG.

ii) The other reason might be that the two processes, “flip-flop” and “projection”, cannot be mediated by one bacterial ABC transporter but need to be shared by two ABC transporter systems.

4. Human ABC proteins in xenobiotic transport

In daily life, we are exposed to various hydrophobic compounds in food and the environment that are apt to pass through the lipid bilayer freely and penetrate our body; inconveniently, many of them have toxic effects. Therefore, our body has to deal with numerous hydrophobic compounds in food and the environment; however, it is impossible to cover the intestine epithelia with cell walls, like bacteria as described in the previous chapters, or to express a huge number of membrane transporters which excrete each hydrophobic toxic compound with high efficiency. Animals have developed mainly two strategies to cope with these hydrophobic substances. One strategy is that hydrophobic toxic substances are conjugated with glutathione, glucuronate, or sulfate, whereby they become more hydrophilic and detoxified. At the same time, these markings make toxic compounds much easier to recognize by transporters. Several ABCC subfamily proteins (MRPs) are involved in transporting these marked compounds out of cells [23-27]. The marked substrates, being hydrophilic, probably directly enter the transporter from cytosol and exit directly to an exogenous environment, as in model (c) in Fig. 1. This pathway is very efficient but has an intrinsic defect, because toxic substances need to be taken into cells to be marked.

The other strategy is to recognize numerous hydrophobic compounds while passing through

the plasma membrane and to excrete them directly out of cells. This is accomplished mainly by two ABC proteins MDR1 and ABCG2 [9,28]. They prevent the absorption of lipophilic toxic compounds of various structures from the intestine, and expels them to bile and urine from the liver and kidney, respectively, although their binding affinities are rather low and in the micromolar range.

The substrates are predicted to enter the transporter from the membrane. Calcein-AM, added to the medium, is efficiently excreted by MDR1 out of cells before being hydrolyzed by esterases in cytosol [29]. Because calcein, the hydrolyzed product, is not the substrate for MDR1, calcein-AM must be recognized while passing through the membrane by MDR1. It is not clear whether substrates exit the transporter to the outer leaflet, as in model (a) in Fig. 1, or directly to an exogenous environment as in model (b); however, because lipophilic compounds readily diffuse inwardly again, it may be necessary to expel them directly to some acceptors, such as albumin, in an exogenous environment for efficient outward transport, as in model (b).

5. ABCB4 in PC transport

Bile formation is important for cholesterol excretion from the body as well as cholesterol absorption from the intestine, and thus it is critical for overall cholesterol homeostasis. Several ABC proteins, expressed on the apical membranes of hepatocytes, are involved in the secretion of bile salts, phospholipids and cholesterol, and thus in canalicular bile formation. ABCB4, also called MDR3 in humans or MDR2 in mice, is essential for the secretion of phosphatidylcholine (PC) into bile [10]. ABCB11, also called a bile salt export pump or a sister of P-glycoprotein,

secretes bile salts into bile [30]. ABCG5 and ABCG8 are the main transporters for the secretion of biliary cholesterol [31].

The function of ABCB4 is required for proper bile formation. Mice with homozygous disruption of the *Abcb4* gene show an almost complete absence of PC in their bile [10], which causes segmental biliary strictures due to periductal fibrosis, fibro-obliteration of bile ducts, and spontaneous gallstone formation. Human ABCB4 mutations result in a wide spectrum of phenotypes, ranging from progressive familial intrahepatic cholestasis type 3 to adult cholestatic liver disorders, characterized by elevated γ -glutamyl transpeptidase levels. The primary function of biliary phospholipid excretion is to protect the membranes of cells facing the biliary tree against bile salts via forming mixed micelles [32].

ABCB4 has an amino acid sequence with 76% identity and 86% similarity to that of MDR1, although ABCB4 does not confer drug resistance. *Abcb4* knockout mice do not excrete any phospholipid into bile, despite the high expression of MDR1 on the canalicular membranes of hepatocytes. MDR1 has quite low, if any, ability to mediate phospholipid excretion, although it actively transports an enormously broad range of compounds to bile. ABCB4 is predicted to transport PC from the inner to the outer leaflet of the canalicular membrane in the “flip-flop” manner, as in model (a) in Fig. 1 and to make PC available for extraction into the canalicular lumen by bile salts [33-35]. ABCB4 exclusively translocates a fluorescent short-chain PC analog containing a 7-nitro-2,1,3-benzoxadiazol group (C₆-NBD-PC) [34]. Interestingly, MDR1 translocates not only C₆-NBD-PC but also C₆-NBD-PE [34]. It has been reported that both

MDR1 and ABCB4 confer resistance to aureobasidin A, an antifungal cyclic depsipeptide antibiotic, when expressed in yeast [36]. The resistance of yeast cells to aureobasidin A conferred by ABCB4 can be overcome by vinblastine, verapamil, and cyclosporin A. Furthermore, human ABCB4 expressed in LLC-PK1 cells mediated transepithelial transport of C₆-NBD-PC, digoxin, paclitaxel, daunorubicin, vinblastine, and ivermectin, although the transport rate is low for most drugs, and the transport is also inhibited by vinblastine, verapamil, and cyclosporin A [37]; therefore, it is conceivable that MDR1 and ABCB4 share conserved domains for substrate recognition and a conserved process to transport substrates, although their physiological roles are quite different.

We reported that HEK293 cells stably expressing human ABCB4 excrete phospholipids (preferentially PC) and cholesterol when bile salts are added to the medium [38]. Because MDR1 or the ATPase-deficient mutant of ABCB4 did not mediate lipid excretion, and no lipid release was observed from host cells even in the presence of bile salts, excretion of PC and cholesterol is directly mediated by ABCB4 in an ATP-dependent manner. *Abcg5/g8*^{-/-} mice have ~80% reduced biliary cholesterol excretion [39]. Because more than 20% of biliary cholesterol excretion was still observed, ABCB4 might also contribute to the secretion of biliary cholesterol in some degree. Sodium taurocholate (NaTC) below the cmc promotes ABCB4-mediated lipid efflux, suggesting that bile salt monomers support lipid efflux. The crystal structure of Sav1866, a bacterial homolog of ABCB1, has been reported [40]. The predicted outward-facing conformation contains a central cavity that is shielded from the inner leaflet of the lipid bilayer

and from the cytoplasm, but exposed to the outer leaflet and the extracellular space. Bile salt monomers may associate with phospholipid and cholesterol in the cavity of ABCB4, and then induce the formation of a mixed bile salt/phospholipid/cholesterol micelle, which is released into the extracellular space. The association of bile salt monomers with phospholipid and cholesterol would reduce the activation energy required to move substrates from the cavity of ABCB4 to the aqueous environment; therefore, PC, entering from the membrane, may exit directly to an exogenous environment as in model (b), as proposed for MDR1 in the previous chapter.

6. ABCA1 in HDL formation

Cholesterol is a key component of the cell membrane and is required for cell proliferation; however, excess accumulation of cholesterol is toxic for cells and its excess deposition in peripheral tissues causes arteriosclerosis. Excess cholesterol is transported to the liver, where it is catabolized to bile acids, as high density lipoprotein (HDL) known as good cholesterol. Because cholesterol is not catabolized in peripheral tissues, HDL formation is the only pathway by which excess cholesterol is removed from peripheral cells. At least 50 mutations, including 23 missense, and 21 insertions or deletions, have been identified in the *ABCA1* gene, leading to Tangier disease and familial hypoalphalipoproteinemia, in which patients have a near absence or decrease of circulating HDL, prominent cholesterol-ester accumulation in tissue macrophages, and premature atherosclerotic vascular disease [41-45]. More than 15 mutations examined showed a high correlation between phospholipids, preferentially PC, and cholesterol efflux [46-48], indicating that ABCA1 influences the efflux of both PC and free cholesterol. ABCA1

mediates the secretion of both types of lipids to an extracellular acceptor in the plasma, apolipoprotein A-I (apoA-I), to form HDL [48,49], and the activity of ABCA1 is highly regulated at the transcriptional level and also at the post-translational level [50-56].

7. Models for ABCA1-mediated HDL formation

Several models have been proposed for the mechanism of ABCA1-mediated HDL formation:

i) A two-step process model: ABCA1 first mediates PC efflux to apoA-I, and this apoA-I-PC complex accepts cholesterol in an ABCA1-independent manner; ii) a concurrent process model: PC and cholesterol efflux by ABCA1 to apoA-I are coupled to each other; and iii) the third model is that ABCA1 generates a specific apoA-I binding site on the plasma membrane and subsequent translocation of PC and cholesterol to apoA-I. When it was proposed by Fielding, P. E. *et al.* [57] and Wang, N. *et al.* [58], the two-step model looked most plausible, because photoactive PC could be cross-linked with ABCA1 whereas direct binding of photoactive cholesterol to ABCA1 could not be detected [58]. However, analysis of functions of ABCA7 raised questions about this model. Human ABCA7, which has the highest homology (66.1%) to ABCA1, mediates the apoA-I-dependent efflux of PC and cholesterol, just as ABCA1 [59]; however, human ABCA7 mediates cholesterol release much less efficiently than ABCA1 [60], and PC but not cholesterol are loaded onto apoA-I by mouse ABCA7 [61]. These results cannot be explained by the two-step process model. Instead, they suggest that ABCA1 have higher affinity for cholesterol to transport than ABCA7 does, and are consistent with the concurrent process model [62].

The third model was originally proposed by Chimini *et al.* [63]: ABCA1 mediates the

translocation of phosphatidylserine (PS) to the outer leaflet, and extracellular exposure of PS promotes apoA-I binding to the cell surface and subsequent translocation of PC and cholesterol to apoA-I. Landry *et al.* [64] reported that ABCA1 re-organizes the plasma membrane and generates loosely packed domains, which facilitate apoA-I association with cells and, subsequently, PC and cholesterol acquisition by apoA-I to form nascent HDL particles. It is still not clear what fraction of lipids is directly loaded onto apoA-I by ABCA1 and what fraction of lipids on apoA-I is indirectly acquired from membranes.

8. Four-step model for ABCA1-mediated HDL formation

Mass and TLC analyses revealed that ABCG1 and ABCA1 secrete several species of sphingomyelin (SM) and PC, and SMs were preferentially secreted by ABCG1, whereas PCs were preferentially secreted by ABCA1 [65]. Thus, ABCA1 and ABCB4 mediate the transport of the same substrates, cholesterol and PC, but their extracellular acceptors are different. ABCB4 mediates the secretion of PC and cholesterol to bile salts as acceptors, but cannot secrete lipids in the presence of apoA-I. On the other hand, cholesterol secretion by ABCA1 is strongly dependent on the presence of lipid-free apoA-I [66,67]. ABCA1 and other members of the ABCA subclass are distinguished from other ABC transporter subclasses by the presence of large extracellular domains (ECDs) [68,69] (Fig. 3). Hozoji *et al.* revealed that two intramolecular disulfide bonds are formed between ECD1 and ECD2 of ABCA1, and these two disulfide bonds are necessary for apoA-I binding and HDL formation [70]. Direct interaction between ABCA1 and apoA-I has been shown via cross-linking experiments [46,66,67,71], even with a cross-linker

as small as 3 Å [72]. These results indicated that the direct binding of apoA-I to ABCA1 is an essential step in HDL formation.

To analyze the differences between ABCA1 and ABCB4, we examined whether NaTC serves as an acceptor for lipids secreted by ABCA1, and surprisingly found that NaTC extracts both cholesterol and PC as efficiently as apoA-I from cells expressing ABCA1 [73]. NaTC did not extract lipids from HEK293 host cells, suggesting that NaTC functions as a lipid acceptor for ABCA1, which mimics apoA-I. The NaTC-dependent efflux of cholesterol and PC is not physiologically relevant, because it is unlikely that the plasma concentration of bile acids reaches 1 mM; however, this system allowed us to analyze the role of apoA-I and dissect the steps in HDL formation.

Because apoA-I does not bind to cells expressing ABCA1-MM, a mutant in which the Walker A lysines in both nucleotide binding domains are substituted by methionines, apoA-I binding is an ATP-dependent process [73,74]. The Tangier mutation W590S, one amino acid substitution in the first ECD, impairs HDL formation [46,48]. We analyzed the function of this Tangier mutant in detail, and found that this mutation greatly decreases NaTC-dependent cholesterol and PC efflux [73]. ABCA1-W590S interacts in a normal manner with ATP [48]. The W590S mutation may abolish the coupling of ATP-induced conformational changes of ABCA1 to lipid translocation. Interestingly, the kinetics of apoA-I binding to cells expressing ABCA1-W590S were similar to those for cells expressing wild-type ABCA1[73], consistent with a previous report showing that the W590S mutation does not impair apoA-I binding

[46,47,71,75]; however, the W590S mutation delayed the dissociation of apoA-I from ABCA1[73].

From these results, we propose that HDL formation mediated by ABCA1 consists of at least four separate steps (Fig. 3): **apoA-I binding step**, ATP binding and/or hydrolysis causes conformational changes within ECDs of ABCA1, to which apoA-I directly binds; **translocation step**, lipid translocation by ABCA1, which is apoA-I-independent; **loading step**, lipid loading of apoA-I bound to ABCA1; and **dissociation step**, dissociation of lipid-loaded apoA-I from ABCA1. In this model, the apoA-I binding step and lipid translocation step, both of which are mediated by ABCA1 in an ATP-dependent manner, are separable. The conformational transition of apoA-I caused by lipid loading probably facilitates the dissociation of apoA-I from ABCA1. ApoA-I undergoes a conformational transition in response to lipid [76], and lipidated apoA-I does not interact with ABCA1 [66,77]. Because the W590S mutation impairs translocation step, apoA-I may remain in lipid-free conformation, which has high affinity to ABCA1.

9. Flip-flop, efflux, or projection

As described in the previous chapter, both ABCA1 and ABCB4 mediate the efflux of PC and cholesterol to NaTC as a lipid acceptor in an ATP-dependent manner; therefore, it is conceivable to predict that they share conserved domains for substrate recognition and conserved process to transport substrates. PC and cholesterol, entering from the membrane, may exit directly to an exogenous environment as in model (b), as proposed for MDR1 and ABCB4.

ABCG1, half-type ABC proteins, functions as a homodimer and transports cholesterol to

nascent HDL to generate mature HDL. ABCG1 mediates the efflux of cholesterol and phospholipids, preferentially SM [65]. The ABCG1-mediated efflux of cholesterol and SM is dependent on the cellular SM level, because lipid efflux by ABCG1 was reduced in a mutant CHO-K1 cell line, LY-A, in which the cellular sphingomyelin level is reduced due to a mutation of ceramide transfer protein CERT [78]. In contrast, the ABCA1-mediated efflux of cholesterol and PC is enhanced in LY-A cells [79]. These results indicate that the substrate specificity of ABCA1 and ABCG1 is different; ABCA1 prefers PC and ABCG1 prefers sphingomyelin. Alternatively, it is possible that ABCA1 preferentially functions in non-raft domains, which contain more PC than SM, and ABCG1 preferentially functions in raft domains, where SM is concentrated, in the plasma membrane.

As SM is synthesized in the lumen of trans-Golgi, and transferred by vesicular transport, SM mainly exists in the outer leaflet. Substrate binding sites of ABCG1 could exist in the outer leaflet, and lipids could enter the binding site mainly from the outer leaflet of the plasma membrane. If this is the case, the outward transport process mediated by ABCG1 would follow model (d) in Fig. 1, in which the substrate is “projected” from the outer leaflet of the membrane without transmembrane movement, as LolCDE. PC also preferentially exists in the outer leaflet of the plasma membrane. Does this suggest that the outward transport process mediated by ABCA1 follows the model (d), as predicted for LolCDE? The four-step model for HDL formation mediated by ABCA1 and apoA-I (Fig. 3) looks similar to the model for the release of the lipoprotein from the inner membrane mediated by LolCDE and LolA (Fig. 2c). Both

apoA-I and LolA directly bind to ABCA1 and LolCDE, respectively. Both ABCA1 and LolCDE move the substrate even in the absence of apoA-I or LolA and load substrates to their acceptors in an ATP-dependent manner. The main difference between the functions of ABCA1 and LolCDE is that the produced nascent HDL consists of more than 200 lipid molecules and two apoA-I proteins, while the LolA/lipoprotein complex in the periplasm consists of one lipoprotein molecule and one LolA protein. Further studies are needed to reveal the mechanism of HDL formation by ABCA1.

From a mechanistic aspect, the alternative access model is generally accepted for the mechanism of most transport; however, the following mechanism may be more appropriate for the projection-type transporter; lipophilic substrates enter the binding site of the transporter from membranes. Conformational change, as in the motion of TM α -helix bundles, caused by ATP binding or hydrolysis makes the binding sites hydrophilic, which facilitates the dissociation of substrates from the binding site to the external environment or directly to the acceptor. The substrates might enter the binding site of the transporter either from the outer or inner leaflet of membranes. Does outward transport by the multi-drug transporter MDR1, highly homologous to ABCB4, also follow this process? We may be able to uncover this mystery by comparing MDR1, ABCB4, ABCA1, and other ABC proteins. 3D-structural analyses of eukaryote drug-transporting and lipid-transporting ABC proteins are required.

ACKNOWLEDGEMENTS

This study was supported by a Grant-in-aid for Scientific research (S) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Japan New Energy and Industrial Technology Development Organization (NEDO), and the World Premier International Research Center Initiative, MEXT, Japan.

References

- [1] Chen, C., Chin, J.E., Ueda, K., Clark, D.P., Pastan, I., Gottesman, M.M. and Roninson, I.B. (1986). Internal duplication and homology with bacterial transport proteins in the *mdr1* (P-glycoprotein) gene from multidrug-resistant human cells. *Cell* 47, 381-389.
- [2] Ueda, K., Cardarelli, C., Gottesman, M.M. and Pastan, I. (1987). Expression of a full-length cDNA for the human "MDR1" gene confers resistance to colchicine, doxorubicin, and vinblastine. *Proc Natl Acad Sci U S A* 84, 3004-8.
- [3] Ueda, K., Cornwell, M.M., Gottesman, M.M., Pastan, I., Roninson, I.B., Ling, V. and Riordan, J.R. (1986). The *mdr1* gene, responsible for multidrug-resistance, codes for P-glycoprotein. *Biochem. Biophys. Res. Commun.* 141, 956-962.
- [4] Juliano, R.L. and Ling, V. (1976). A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim. Biophys. Acta* 455, 152-162.
- [5] Gottesman, M.M. and Pastan, I. (1993). Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu. Rev. Biochem.* 62, 385-427.
- [6] Juranka, P.F., Zastawny, R.L. and Ling, V. (1989). P-glycoprotein: multidrug-resistance and a superfamily of membrane-associated transport proteins. *FASEB J.* 3, 2583-2592.
- [7] Tsuruo, T. and Tomida, A. (1995). Multidrug resistance. *Anticancer Drugs* 6, 213-8.
- [8] Schinkel, A.H. (1997). The physiological function of drug-transporting P-glycoproteins. *Semin Cancer Biol* 8, 161-70.
- [9] Giacomini, K.M. et al. (2010). Membrane transporters in drug development. *Nat Rev Drug Discov* 9, 215-36.
- [10] Smit, J.J. et al. (1993). Homozygous disruption of the murine *mdr2* P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. *Cell* 75, 451-62.
- [11] Higgins, C.F. and Gottesman, M.M. (1992). Is the multidrug transporter a flippase? *Trends Biochem. Sci.* 17, 18-21.
- [12] Kimura, Y., Kodan, A., Matsuo, M. and Ueda, K. (2007). Cholesterol fill-in model: mechanism for substrate recognition by ABC proteins. *J Bioenerg Biomembr* 39, 447-52.
- [13] Kimura, Y., Morita, S.Y., Matsuo, M. and Ueda, K. (2007). Mechanism of multidrug recognition by

MDR1/ABCB1. *Cancer Sci* 98, 1303-1310.

- [14] Voelker, D.R. (2009). Genetic and biochemical analysis of non-vesicular lipid traffic. *Annu Rev Biochem* 78, 827-56.
- [15] Tokuda, H. (2009). Biogenesis of outer membranes in Gram-negative bacteria. *Biosci Biotechnol Biochem* 73, 465-73.
- [16] Narita, S., Tanaka, K., Matsuyama, S. and Tokuda, H. (2002). Disruption of *lolCDE*, encoding an ATP-binding cassette transporter, is lethal for *Escherichia coli* and prevents release of lipoproteins from the inner membrane. *J Bacteriol* 184, 1417-22.
- [17] Yakushi, T., Masuda, K., Narita, S., Matsuyama, S. and Tokuda, H. (2000). A new ABC transporter mediating the detachment of lipid-modified proteins from membranes. *Nat Cell Biol* 2, 212-8.
- [18] Zhou, Z., White, K.A., Polissi, A., Georgopoulos, C. and Raetz, C.R. (1998). Function of *Escherichia coli* *MsbA*, an essential ABC family transporter, in lipid A and phospholipid biosynthesis. *J Biol Chem* 273, 12466-75.
- [19] Raetz, C.R. and Whitfield, C. (2002). Lipopolysaccharide endotoxins. *Annu Rev Biochem* 71, 635-700.
- [20] Sperandio, P., Lau, F.K., Carpentieri, A., De Castro, C., Molinaro, A., Deho, G., Silhavy, T.J. and Polissi, A. (2008). Functional analysis of the protein machinery required for transport of lipopolysaccharide to the outer membrane of *Escherichia coli*. *J Bacteriol* 190, 4460-9.
- [21] Ruiz, N., Gronenberg, L.S., Kahne, D. and Silhavy, T.J. (2008). Identification of two inner-membrane proteins required for the transport of lipopolysaccharide to the outer membrane of *Escherichia coli*. *Proc Natl Acad Sci U S A* 105, 5537-42.
- [22] Narita, S. and Tokuda, H. (2009). Biochemical characterization of an ABC transporter *LptBFGC* complex required for the outer membrane sorting of lipopolysaccharides. *FEBS Lett* 583, 2160-4.
- [23] Ishikawa, T. (1992). The ATP-dependent glutathione S-conjugate export pump. *Trends. Biochem. Sci.* 17, 463-468.
- [24] Borst, P., Evers, R., Kool, M. and Wijnholds, J. (2000). A family of drug transporters: the multidrug resistance-associated proteins. *J Natl Cancer Inst* 92, 1295-302.
- [25] Konig, J., Nies, A.T., Cui, Y., Leier, I. and Keppler, D. (1999). Conjugate export pumps of the multidrug resistance protein (MRP) family: localization, substrate specificity, and MRP2-mediated drug resistance. *Biochim Biophys Acta* 1461, 377-94.
- [26] Leslie, E.M., Deeley, R.G. and Cole, S.P. (2001). Toxicological relevance of the multidrug resistance protein 1, MRP1 (ABCC1) and related transporters. *Toxicology* 167, 3-23.
- [27] Schinkel, A.H. and Jonker, J.W. (2003). Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. *Adv Drug Deliv Rev* 55, 3-29.
- [28] Sarkadi, B., Homolya, L., Szakacs, G. and Varadi, A. (2006). Human multidrug resistance ABCB and ABCG transporters: participation in a chemoinnity defense system. *Physiol Rev* 86, 1179-236.
- [29] Homolya, L., Hollo, Z., Germann, U.A., Pastan, I., Gottesman, M.M. and Sarkadi, B. (1993). Fluorescent cellular indicators are extruded by the multidrug resistance protein. *J. Biol. Chem.*

- [30] Wang, R. et al. (2001). Targeted inactivation of sister of P-glycoprotein gene (spgp) in mice results in nonprogressive but persistent intrahepatic cholestasis. *Proc Natl Acad Sci U S A* 98, 2011-6.
- [31] Berge, K.E. et al. (2000). Accumulation of dietary cholesterol in sitosterolemia caused by mutations in adjacent ABC transporters. *Science* 290, 1771-5.
- [32] Oude Elferink, R.P. and Paulusma, C.C. (2007). Function and pathophysiological importance of ABCB4 (MDR3 P-glycoprotein). *Pflugers Arch* 453, 601-10.
- [33] Smith, A.J., Timmermans-Hereijgers, J.L., Roelofsen, B., Wirtz, K.W., Blitterswijk, W.J.v., Smit, J.J., Schinkel, A.H. and Borst, P. (1994). The human MDR3 P-glycoprotein promotes translocation of phosphatidylcholine through the plasma membrane of fibroblasts from transgenic mice. *FEBS Lett* 354, 263-266.
- [34] van Helvoort, A., Smith, A.J., Sprong, H., Fritzsche, I., Schinkel, A.H., Borst, P. and van Meer, G. (1996). MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 P-glycoprotein specifically translocates phosphatidylcholine. *Cell* 87, 507-17.
- [35] Borst, P., Zelcer, N. and van Helvoort, A. (2000). ABC transporters in lipid transport. *Biochim Biophys Acta* 1486, 128-44.
- [36] Kino, K., Taguchi, Y., Yamada, K., Komano, T. and Ueda, K. (1996). Aureobasidin A, an antifungal cyclic depsipeptide antibiotic, is a substrate for both human MDR1 and MDR2/P-glycoproteins. *FEBS Lett* 399, 29-32.
- [37] Smith, A.J. et al. (2000). MDR3 P-glycoprotein, a phosphatidylcholine translocase, transports several cytotoxic drugs and directly interacts with drugs as judged by interference with nucleotide trapping. *J Biol Chem* 275, 23530-9.
- [38] Morita, S.Y., Kobayashi, A., Takanezawa, Y., Kioka, N., Handa, T., Arai, H., Matsuo, M. and Ueda, K. (2007). Bile salt-dependent efflux of cellular phospholipids mediated by ATP binding cassette protein B4. *Hepatology* 46, 188-99.
- [39] Yu, L., Hammer, R.E., Li-Hawkins, J., Von Bergmann, K., Lutjohann, D., Cohen, J.C. and Hobbs, H.H. (2002). Disruption of Abcg5 and Abcg8 in mice reveals their crucial role in biliary cholesterol secretion. *Proc Natl Acad Sci U S A* 99, 16237-42.
- [40] Dawson, R.J. and Locher, K.P. (2006). Structure of a bacterial multidrug ABC transporter. *Nature* 443, 180-5.
- [41] Singaraja, R.R., Brunham, L.R., Visscher, H., Kastelein, J.J. and Hayden, M.R. (2003). Efflux and atherosclerosis: the clinical and biochemical impact of variations in the ABCA1 gene. *Arterioscler Thromb Vasc Biol* 23, 1322-32.
- [42] Oram, J.F. and Vaughan, A.M. (2006). ATP-Binding cassette cholesterol transporters and cardiovascular disease. *Circ Res* 99, 1031-43.
- [43] Bodzioch, M. et al. (1999). The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. *Nat Genet.* 22, 347-351.
- [44] Brooks-Wilson, A. et al. (1999). Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency. *Nat Genet.* 22, 336-345.
- [45] Rust, S. et al. (1999). Tangier disease is caused by mutations in the gene encoding ATP-binding

- cassette transporter 1. *Nat Genet.* 22, 352-355.
- [46] Fitzgerald, M.L., Morris, A.L., Rhee, J.S., Andersson, L.P., Mendez, A.J. and Freeman, M.W. (2002). Naturally occurring mutations in ABCA1's largest extracellular loops can disrupt its direct interaction with apolipoprotein A-I. *J Biol Chem* 277, 33178-87.
 - [47] Singaraja, R.R. et al. (2006). Specific mutations in ABCA1 have discrete effects on ABCA1 function and lipid phenotypes both in vivo and in vitro. *Circ Res* 99, 389-97.
 - [48] Tanaka, A.R. et al. (2003). Effects of mutations of ABCA1 in the first extracellular domain on subcellular trafficking and ATP binding/hydrolysis. *J Biol Chem* 278, 8815-8819.
 - [49] Yokoyama, S. (2000). Release of cellular cholesterol: molecular mechanism for cholesterol homeostasis in cells and in the body. *Biochim. Biophys. Acta* 1529, 231-244.
 - [50] Hozoji, M., Munehira, Y., Ikeda, Y., Makishima, M., Matsuo, M., Kioka, N. and Ueda, K. (2008). Direct interaction of nuclear liver X receptor-beta with ABCA1 modulates cholesterol efflux. *J Biol Chem* 283, 30057-63.
 - [51] Munehira, Y. et al. (2004). Alpha1-syntrophin modulates turnover of ABCA1. *J Biol Chem* 279, 15091-5.
 - [52] Feng, B. and Tabas, I. (2002). ABCA1-mediated cholesterol efflux is defective in free cholesterol-loaded macrophages. Mechanism involves enhanced ABCA1 degradation in a process requiring full NPC1 activity. *J Biol Chem* 277, 43271-80.
 - [53] Hassan, H.H., Bailey, D., Lee, D.Y., Iatan, I., Hafiane, A., Ruel, I., Krimbou, L. and Genest, J. (2008). Quantitative Analysis of ABCA1-dependent Compartmentalization and Trafficking of Apolipoprotein A-I: IMPLICATIONS FOR DETERMINING CELLULAR KINETICS OF NASCENT HIGH DENSITY LIPOPROTEIN BIOGENESIS. *J Biol Chem* 283, 11164-75.
 - [54] Lu, R., Arakawa, R., Ito-Osumi, C., Iwamoto, N. and Yokoyama, S. (2008). ApoA-I facilitates ABCA1 recycle/accumulation to cell surface by inhibiting its intracellular degradation and increases HDL generation. *Arterioscler Thromb Vasc Biol* 28, 1820-4.
 - [55] Okuhira, K., Fitzgerald, M.L., Sarracino, D.A., Manning, J.J., Bell, S.A., Goss, J.L. and Freeman, M.W. (2005). Purification of ATP-binding cassette transporter A1 and associated binding proteins reveals the importance of beta1-syntrophin in cholesterol efflux. *J Biol Chem* 280, 39653-64.
 - [56] Wang, N., Chen, W., Linsel-Nitschke, P., Martinez, L.O., Agerholm-Larsen, B., Silver, D.L. and Tall, A.R. (2003). A PEST sequence in ABCA1 regulates degradation by calpain protease and stabilization of ABCA1 by apoA-I. *J Clin Invest* 111, 99-107.
 - [57] Fielding, P.E., Nagao, K., Hakamata, H., Chimini, G. and Fielding, C.J. (2000). A two-step mechanism for free cholesterol and phospholipid efflux from human vascular cells to apolipoprotein A-1. *Biochemistry* 39, 14113-14120.
 - [58] Wang, N., Silver, D.L., Thiele, C. and Tall, A.R. (2001). ATP-binding cassette transporter A1 (ABCA1) functions as a cholesterol efflux regulatory protein. *J Biol Chem* 276, 23742-7.
 - [59] Abe-Dohmae, S., Ikeda, Y., Matsuo, M., Hayashi, M., Okuhira, K., Ueda, K. and Yokoyama, S. (2004). Human ABCA7 Supports Apolipoprotein-mediated Release of Cellular Cholesterol and Phospholipid to Generate High Density Lipoprotein. *J Biol Chem* 279, 604-611.
 - [60] Hayashi, M., Abe-Dohmae, S., Okazaki, M., Ueda, K. and Yokoyama, S. (2005). Heterogeneity of

- high density lipoprotein generated by ABCA1 and ABCA7. *J Lipid Res* 46, 1703-11.
- [61] Wang, N., Lan, D., Gerbod-Giannone, M., Linsel-Nitschke, P., Jehle, A.W., Chen, W., Martinez, L.O. and Tall, A.R. (2003). ATP-binding Cassette Transporter A7 (ABCA7) Binds Apolipoprotein A-I and Mediates Cellular Phospholipid but Not Cholesterol Efflux. *J Biol Chem* 278, 42906-12.
 - [62] Smith, J.D., Le Goff, W., Settle, M., Brubaker, G., Waelde, C., Horwitz, A. and Oda, M.N. (2004). ABCA1 mediates concurrent cholesterol and phospholipid efflux to apolipoprotein A-I. *J Lipid Res* 45, 635-44.
 - [63] Hamon, Y. et al. (2000). ABC1 promotes engulfment of apoptotic cells and transbilayer redistribution of phosphatidylserine. *Nat Cell Biol.* 2, 399-406.
 - [64] Landry, Y.D., Denis, M., Nandi, S., Bell, S., Vaughan, A.M. and Zha, X. (2006). ABCA1 expression disrupts raft membrane microdomains through its ATPase-related functions. *J Biol Chem* 281, 36091-36101.
 - [65] Kobayashi, A. et al. (2006). Efflux of sphingomyelin, cholesterol, and phosphatidylcholine by ABCG1. *J Lipid Res* 47, 1791-802.
 - [66] Wang, N., Silver, D., Costet, P. and Tall, A. (2000). Specific binding of ApoA-I, enhanced cholesterol efflux, and altered plasma membrane morphology in cells expressing ABC1. *J Biol Chem* 275, 33053-33058.
 - [67] Oram, J., Lawn, R., Garvin, M. and Wade, D. (2000). ABCA1 is the cAMP-inducible apolipoprotein receptor that mediates cholesterol secretion from macrophages. *J Biol Chem* 275, 34508-34511.
 - [68] Peelman, F. et al. (2003). Characterization of the ABCA transporter subfamily: identification of prokaryotic and eukaryotic members, phylogeny and topology. *J Mol Biol* 325, 259-74.
 - [69] Tanaka, A.R. et al. (2001). Human ABCA1 Contains a Large Amino-Terminal Extracellular Domain Homologous to an Epitope of Sjogren's Syndrome. *Biochem Biophys Res Commun.* 283, 1019-25.
 - [70] Hozoji, M., Kimura, Y., Kioka, N. and Ueda, K. (2009). Formation of two intramolecular disulfide bonds is necessary for ApoA-I-dependent cholesterol efflux mediated by ABCA1. *J Biol Chem* 284, 11293-300.
 - [71] Fitzgerald, M.L., Morris, A.L., Chroni, A., Mendez, A.J., Zannis, V.I. and Freeman, M.W. (2004). ABCA1 and amphipathic apolipoproteins form high-affinity molecular complexes required for cholesterol efflux. *J Lipid Res* 45, 287-94.
 - [72] Chroni, A., Liu, T., Fitzgerald, M.L., Freeman, M.W. and Zannis, V.I. (2004). Cross-linking and lipid efflux properties of apoA-I mutants suggest direct association between apoA-I helices and ABCA1. *Biochemistry* 43, 2126-39.
 - [73] Nagao, K., Zhao, Y., Takahashi, K., Kimura, Y. and Ueda, K. (2009). Sodium taurocholate-dependent lipid efflux by ABCA1: effects of W590S mutation on lipid translocation and apolipoprotein A-I dissociation. *J Lipid Res* 50, 1165-72.
 - [74] Chambenoit, O., Hamon, Y., Marguet, D., Rigneault, H., Rosseneu, M. and Chimini, G. (2001). Specific docking of apolipoprotein A-I at the cell surface requires a functional ABCA1 transporter. *J. Biol. Chem.* 276, 9955-60.
 - [75] Rigot, V., Hamon, Y., Chambenoit, O., Alibert, M., Duverger, N. and Chimini, G. (2002). Distinct

- sites on ABCA1 control distinct steps required for cellular release of phospholipids. *J. Lipid Res.* 43, 2077-86.
- [76] Davidson, W.S. and Thompson, T.B. (2007). The structure of apolipoprotein A-I in high density lipoproteins. *J Biol Chem* 282, 22249-53.
- [77] Mulya, A., Lee, J.Y., Gebre, A.K., Thomas, M.J., Colvin, P.L. and Parks, J.S. (2007). Minimal lipidation of pre-beta HDL by ABCA1 results in reduced ability to interact with ABCA1. *Arterioscler Thromb Vasc Biol* 27, 1828-36.
- [78] Sano, O., Kobayashi, A., Nagao, K., Kumagai, K., Kioka, N., Hanada, K., Ueda, K. and Matsuo, M. (2007). Sphingomyelin-dependence of cholesterol efflux mediated by ABCG1. *J Lipid Res* 48, 2377-84.
- [79] Nagao, K., Takahashi, K., Hanada, K., Kioka, N., Matsuo, M. and Ueda, K. (2007). Enhanced apoA-I-dependent cholesterol efflux by ABCA1 from sphingomyelin-deficient Chinese hamster ovary cells. *J Biol Chem* 282, 14868-74.

Figure legends

Fig. 1 Four processes predicted for outward lipid transport

(a) The substrates enter the transporter from the inner leaflet and exit from the transporter into the outer leaflet. Substrates could be moved from one substrate binding site to the other in the transporter in a “flip-flop” way. (b) Substrates enter the transporter from the inner leaflet and exit the transporter directly to an exogenous environment. (c) Amphiphilic substrates directly enter the transporter from cytosol and exit directly to an exogenous environment. This is similar to the transport mechanisms for ions and solutes. Substrates are sometimes marked with glutathione, glucuronate, or sulfate. (d) The substrates enter the transporter from the outer leaflet and exit the transporter directly to an exogenous environment. The movement of substrates in this model can be called “projection” to the exogenous environment.

Fig. 2 Bacterial ABC proteins involved in lipoprotein and LPS transport

(a) Lipoprotein transport from the inner to the outer membrane. (b) LPS transport from the inner to the outer membrane. (c) Molecular events involved in lipoprotein release from the inner membrane. Modified from ref 15 with permission from the publisher

Fig. 3 Four-step model of HDL formation mediated by ABCA1

ApoA-I binding step, ATP binding and/or hydrolysis causes conformational changes within ECDs of ABCA1, to which apoA-I directly binds. Translocation step, lipid translocation by ABCA1, which is apoA-I-independent. Loading step, lipid loading of apoA-I bound to ABCA1. Dissociation step, dissociation of lipid-loaded apoA-I from ABCA1. In this model, the apoA-I binding step and lipid translocation step, both of which are mediated by ABCA1 in an ATP-dependent manner, are separable. The conformational transition of apoA-I caused by lipid loading probably facilitates the dissociation of apoA-I from ABCA1.