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Cholesterol fill-in model: mechanism for substrate recognition by ABC proteins

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Abstract Many of the 48 or 49 human ABC proteins are involved in lipid homeostasis and in defence against hydrophobic substances in food and the environment. Defects in their functions cause various diseases, suggesting that they play very important roles in human health; however, the mechanism of how they handle enormous numbers of hydrophobic compounds with various structures and molecular weights, or phospholipids and cholesterol, major components of cellular membranes, is not known. We compared the functions of drug-transporting and lipid-transporting ABC proteins, and found that (1) ABC proteins, either lipid or drug transporters, have a similar substrate binding site which recognizes PL and cholesterol, or drugs and cholesterol; (2) Cholesterol in membranes binds to various ABC proteins together with PL or drugs, and plays an important role in substrate recognition, especially by ABCB1/MDR1, where cholesterol fills the empty space in the substrate binding site when small drugs bind to it. ABC proteins exert very flexible substrate recognition, i.e., one-to-many interaction rather than the conventional rigid one-to-one interaction. We propose calling the mechanism the "cholesterol fill-in model".

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Introduction

In general, physiologically important reactions are considered to be specific and occur with high-affinity binding constant (i.e., in nanomolar order). In contrast, substrate recognition by ABC proteins are not highly specific and binding affinity is in micromolar order in many cases. A question may therefore be raised: "Are reactions in which ABC proteins are involved less important in physiology?" Indeed, knock-out of ABC protein genes hardly affects a mouse's life under normal conditions; however, we now know that defects in many of the 48 or 49 human ABC protein genes cause various diseases in humans (Table 1). These features, not a prerequisite for living but important for human health, are an important key to understand the function of ABC proteins.

In daily life, we are exposed to various hydrophobic compounds in food and the environment, that are apt to go 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 a cell wall, partially resistant against high molecular weight hydrophobic substances, or to express a huge number of membrane transporters which excrete each hydrophobic toxic compound with high efficiency. We have to develop another strategy to cope with hydrophobic substances, and that is ABCB1/MDR1.

Table 1 Human ABC proteins, functions and diseases

Symbol	Gene	Amino acid	Function/phenotype, disease
ABCA subfamily			
ABC1	ABCA1	2261	Cholesterol and phospholipid efflux/HDL deficiency
ABC2	ABCA2	2436	Lipid transport?/abnormal myelin formation
ABC3	ABCA3	1704	Secretion of pulmonary surfactant/surfactant deficiency in newborn
ABCR	ABCA4	2273	Retinoic acid transport/Stargardt disease 1
ABCA7	ABCA7	2146	Phospholipid transport
ABCA12	ABCA12	2595	Lipid transport?/Harlequin ichthyosis
ABCB subfamily			
MDR1, PGY1	ABCB1	1280	Xenobiotics efflux pump/multidrug resistance in cancer
TAP1	ABCB2	808	Transport of antigen peptide into ER lumen/Behçet's disease
TAP2	ABCB3	653	Transport of antigen peptide into ER lumen/Behçet's disease
MDR2/3	ABCB4	1279	Secretion of phosphatidylcholine into bile/intrahepatic cholestasis
ABCB6	ABCB6	842	Transport of porphyrin in mitochondria
ABC7	ABCB7	752	Transport of iron-sulfate complexes in mitochondria
SPGP, BSEP	ABCB11	1321	Bile acid export/intrahepatic cholestasis
ABCC subfamily			
MRP1	ABCC1	1531	Transport of detoxificated xenobiotics/multidrug resistance in cancer
MRP2/cMOAT	ABCC2	1545	Bilirubin export/Dubin–Johnson syndrome
MRP3	ABCC3	1527	Excretion of sulfate and glucuronide metabolites
MRP4	ABCC4	1325	Transport of nucleoside based anti-viral drugs and prostaglandin
MRP5	ABCC5	1437	Transport of cyclic nucleotides and nucleoside monophosphate analogs
MRP6	ABCC6	1503	?/Pseudoxanthoma elasticum
CFTR	ABCC7	1480	Cl^- channel/cystic fibrosis
SUR1	ABCC8	1581	ATP sensitive K^+ channel regulator in pancreatic β-cells/PHHI
SUR2	ABCC9	1549	ATP sensitive K^+ channel regulator in cardiac myocyte
ABCD subfamily			
ALDP	ABCD1	745	Peroxysomal transport of very long fatty acid/adrenoleukodystrophy
ALDR	ABCD2	740	Peroxysomal transport of very long fatty acid/adrenoleukodystrophy
PMP70	ABCD3	659	Peroxysomal transport of very long fatty acid
ABCG subfamily			
ABCG1	ABCG1	678	Transport of cholesterol and phospholipid
ABCP, BCRP	ABCG2	655	Multidrug resistance, specific expression in stem cells
ABCG5	ABCG5	651	Export of phytosterols/sitosterolemia
ABCG8	ABCG8	673	Export of phytosterols/sitosterolemia

ABCB1/MDR1 is expressed in the intestine, liver, kidney, and other organs, and excretes numerous kinds of hydrophobic compounds from molecular weight 300 to 2,000. Since the binding affinities to these compounds are low and usually in the micromolar range, ABCB1/MDR1 can not excrete hydrophobic substances at low concentrations. Another system, such as detoxification by P450 enzymes, would work in those cases.

When we think about the functions of lipid-transporting ABC proteins, the binding affinity of transport substrates can not be high. As phospholipids (PL) and cholesterol are major components (about 20% in both cases) of cellular membranes, "low binding affinity" would be necessary for efficient turnover of the transport cycle. In this minireview, we consider how drug-transporting and lipid-transporting ABC proteins recognize hydrophobic compounds as transport substrates by comparing the functions of several ABC proteins, ABCA1, ABCA7, ABCG1, ABCB1/MDR1, and ABCB4/MDR2.

ABCA1 and ABCA7

It had been considered that serum high-density lipoprotein (HDL) was generated by the concentration-dependent movement of cholesterol from lipid-rich cell membranes to lipid-poor lipoprotein in serum; however, the importance of ABCA1 became obvious by the finding of mutations in the ABCA1 gene from Tangier disease patients in 1999 (Brooks-Wilson et al. 1999; Bodzioch et al. 1999; Rust et al. 1999). When apoA-I is added to the medium of cells expressing ABCA1, cholesterol and PL are released into the medium and nascent HDL particles are generated (Tanaka et al. 2001; Wang et al. 2000). ABCA1 harboring

a Tangier disease mutation does not mediate the release of cholesterol or PL (Tanaka et al. 2003; Fitzgerald et al. 2002), indicating that functional ABCA1 is a prerequisite for the movement of cholesterol and PL from cells to apoA-I; however, it is still controversial whether ABCA1 directly recognizes cholesterol and PL as substrates, and whether ABCA1 "transports" them in an ATP-dependent manner.

To explore the mechanism of ABCA1-mediated HDL formation, we expressed human ABCA1 in insect Sf9 cells and purified it (Takahashi et al. 2006). Purified ABCA1 showed robust ATPase activity when reconstituted in liposomes made of synthetic phosphatidylcholine (PC) or sphingomyelin (SM). ABCA1 showed lower ATPase activity when reconstituted in liposomes containing phosphatidylserine, phosphatidylethanolamine, or phosphatidylglycerol, suggesting that ABCA1 recognizes the choline head group of PL; however, unexpectedly, ATPase activity was reduced by the addition of cholesterol and decreased by 25% in the presence of 20% cholesterol. Interestingly, ATPase activity of ABCG5/ABCG8 complex, which excretes sitosterol and cholesterol into bile, is also suppressed by the addition of cholesterol (Wang et al. 2006). We have not been able to determine whether cholesterol is directly recognized by ABCA1 as a transport substrate.

When we compared the functions of ABCA1 and ABCA7, which has the highest homology (66.1%) to ABCA1, by establishing cells expressing ABCA1 and ABCA7 at various levels, we found the ABCA7 mediates apoA-I dependent cholesterol release with much less efficiency than ABCA1, although PC efflux was comparable (Hayashi et al. 2005). It has also been reported that PL but not cholesterol are loaded onto apoA-I by mouse ABCA7 (Wang et al. 2003). These results are inconsistent with the two-step process model proposed by Fielding et al. (2000) and Wang et al. (2001) that ABCA1 first mediates PL efflux to apoA-I and this apolipoprotein-PL complex accepts cholesterol in an ABCA1-independent manner. It is assumed that ABCA1 and ABCA7 have different substrate specificities for transport, and that cholesterol is one of the transport substrates for ABCA1. The interaction between ABCA1 and cholesterol should be investigated further.

ABCA1 and ABCG1

It was recently proposed that ABCA1 relocates SM from raft to non-raft domains in the plasma membrane and generates more loosely packed domains that facilitate apoA-I association with cells and, consequently, lipid acquisition by apoA-I to form nascent HDL particles (Landry et al. 2006). It was also reported that macrophages from ABCA1-deficient mice exhibited increased lipid rafts on the cell surface (Koseki et al. 2007). In our experiments using purified protein, ATPase activity of ABCA1 was stimulated by SM as well as PC (Takahashi et al. 2006). We then examined whether ABCA1 expression affects the amount or distribution of SM and whether the ABCA1-dependent alteration of SM is required for apoA-I-dependent HDL formation.

In cells, ceramide is transferred from ER to the trans Golgi by ceramide transfer protein CERT, and then SM is synthesized in the Golgi apparatus; therefore, de novo SM synthesis is impaired in LY-A cells, a Chinese hamster ovary (CHO)-K1-derived mutant cell line defect in CERT (Hanada et al. 2003). Indeed, when LY-A cells were cultured in a sphingolipid-deficient medium, SM content in LY-A cells became about 65% of that of LY-A/CERT cells stably transfected with human CERT cDNA.

The expression of ABCA1 can be greatly stimulated by an LXR agonist, TO901317, and an RXR agonist, retinoic acid, and their expression levels were undistinguishable between LY-A and LY-A/CERT cells. Furthermore, a biotinylation experiment suggested that the amount of ABCA1 expressed on the cell surface is also undistinguishable between these cells. When cells were cultured in the sphingolipid-deficient medium, apoA-I-dependent cholesterol and PL efflux by ABCA1 from LY-A cells increased 1.6-fold compared with that from LY-A/CERT cells. There was no difference in cholesterol and PL excretion between the two cell types when cells were cultured in medium containing 10% FBS (Nagao et al. 2007). Furthermore, the addition of SM, which increased cellular SM content by 60–70%, significantly reduced ABCA1-mediated cholesterol efflux. These results suggest that the decrease in SM content in the plasma membrane stimulates cholesterol efflux by ABCA1.

Next, the effect of SM content on ABCG1-mediated cholesterol efflux to HDL was examined (Sano et al. 2007). ABCG1 mediated PL and cholesterol efflux from LY-A/CERT cells as we previously reported (Kobayashi et al. 2006); however, ABCG1 scarcely increased PL or cholesterol efflux from LY-A cells compared with mock transfected cells, when cells were cultured in a sphingolipid-deficient medium. This means that the effects of SM content on the function of ABCA1 and ABCG1 are opposite. In the case of ABCA1, when SM content of the plasma membrane decreased, ABCA1-mediated cholesterol efflux increased. In contrast, when SM content of the plasma membrane decreased, ABCG1-mediated cholesterol efflux decreased.

Furthermore, knockdown of CERT in HeLaS3 cells significantly suppressed ABCG1-mediated cholesterol efflux. These results suggest that the decrease in SM content impairs ABCG1-mediated cholesterol efflux. Mass-spectrometric analysis of lipids secreted into the medium suggested that ABCA1 preferentially secretes PC into the medium in the presence of apoA-I, while ABCG1 preferentially secretes SM into the medium (Kobayashi et al. 2006). SM has high affinity for cholesterol, and

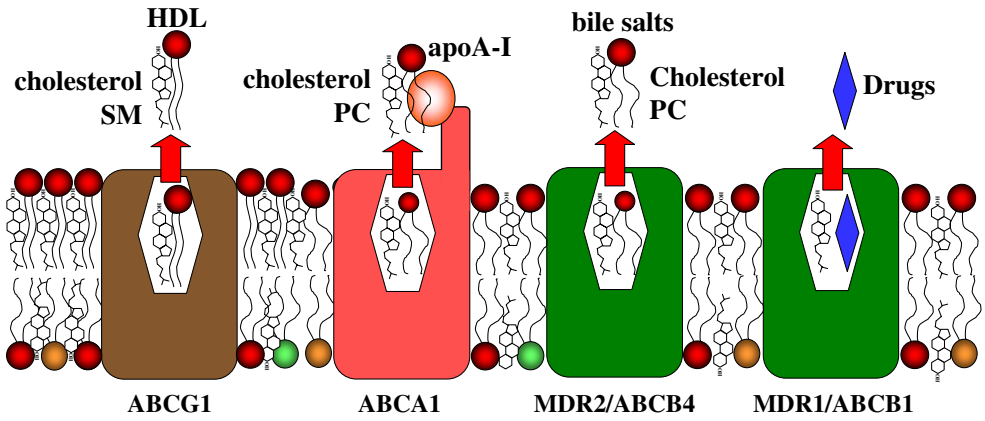

Fig. 1 Cholesterol fill-in model of substrate recognition by ABC proteins. ABCG1 recognizes sphingomyelin and cholesterol, and excretes them together in an ATP-dependent manner. HDL works as an acceptor. ABCA1 recognizes phosphatidylcholine and cholesterol, and excretes them together in an ATP-dependent manner. Lipid-free apoA-I works as an acceptor. ABCB4/MDR2 recognizes phosphatidylcholine and cholesterol, and excretes them together in an ATP-dependent manner. Bile salts work as acceptors. ABCB1/MDR1 recognizes various drugs and cholesterol and excretes only drugs in an ATP-dependent manner. No acceptors are required

tends to form a complex with cholesterol. We assume that ABCG1 has a binding site for SM-cholesterol complex and transports SM-cholesterol complex, while ABCA1 transports PC-cholesterol complex (Fig. 1).

ABCB1/MDR1 and ABCB4/MDR2

ABCB1/MDR1 confers multidrug resistance to cells by excreting various drugs in an ATP-dependent manner (Ueda et al. 1987), and excretes various xenobiotics into bile canaliculi from hepatocytes. In hepatocytes, other ABC proteins, ABCB4/MDR2 and ABCB11/BSEP, are expressed on the same membrane. Although ABCB1/MDR1 and ABCB4/MDR2 share high amino acid sequence homology (76% identity and 86% similarity; van der Bliek et al. 1988), ABCB4/MDR2 has been suggested to excrete PL rather than xenobiotics into bile, and ABCB1/MDR1 does not excrete PL; however, the mechanism of PL efflux has not been characterized in detail.

We established cells expressing ABCB1/MDR1 and ABCB4/MDR2, and found that in the presence of 0.5 mM sodium taurocholate (NaTC), a considerable amount of PL is secreted from ABCB4/MDR2-expressing cells into the medium (Morita et al. 2007). From ABCB1/MDR1-expressing cells, no PL was secreted. Interestingly, cholesterol was also secreted from ABCB4/MDR2-expressing cells in the presence of taurocholate. ABCB4/MDR2 with a mutation in the ATP binding domain did not secrete PL or cholesterol, suggesting that PL and cholesterol secretion is ATP hydrolysis dependent. Mass-spectrometric analysis suggested that PC is the main species secreted by ABCB4/MDR2.

Judging from the high sequence identity, it is conceivable that ABCB4/MDR2 has conserved domains for substrate recognition in common with ABCB1/MDR1. Indeed, it has been reported that both ABCB1/MDR1 and ABCB4/MDR2 confer resistance to aureobasidin A, an antifungal cyclic depsipeptide antibiotic, when expressed in yeast (Kino et al. 1996). The resistance of yeast cells to aureobasidin A conferred by ABCB4/MDR2 can be overcome by vinblastine, verapamil, and cyclosporin A. Transepithelial transport of C6-NBD-PC and digoxin by ABCB4/MDR2 through LLC-PK1 cells is inhibited by vinblastine, verapamil, and cyclosporin A, indicating an interaction between these compounds and ABCB4/MDR2 (Smith et al. 2000). The translocation of NBD-PC in yeast secretory vesicles is mediated by both ABCB1/MDR1 and

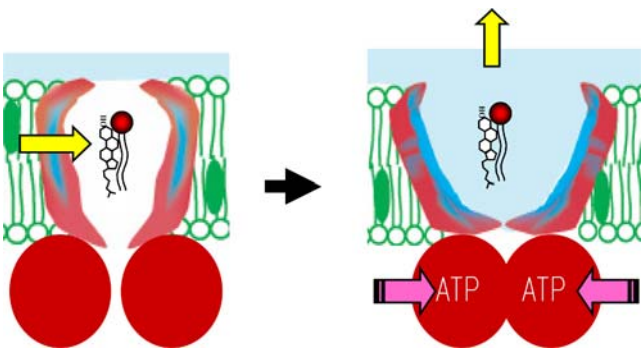

Fig. 2 Substrate may be able to enter the binding site either from the outer leaflet or the inner leaflet. The conformational change, like the motion in TM α-helix bundles, induced by ATP binding, may not cause the substrates to flop but just makes the binding sites hydrophilic, which facilitates the dissociation of the substrates from the binding site to the medium or directly to the acceptor

ABCB4/MDR2 and abrogated by verapamil (Ruetz and Gros 1994). We showed that verapamil almost completely abolished the NaTC-dependent efflux of PL and cholesterol mediated by ABCB4/MDR2 (Morita et al. 2007). These results indicate that ABCB1/MDR1 and ABCB4/MDR2 have quite similar substrate binding domains, however, ABCB1/MDR1 cannot transport PC even in the presence of NaTC, whereas ABCB4/MDR2 can.

Although cholesterol was not secreted from ABCB1/MDR1-expressing cells, we found that cholesterol affects the drug-stimulated ATPase activity of purified ABCB1/MDR1 (Kimura et al. 2007). The binding affinity of drugs, such as rhodamine 123, dexamethasone, verapamil, nicardipine, digoxin, corticosterone, hydrocortisone, and rhodamine B, with a low molecular weight, between 350 and 500, increased in the presence of 20% cholesterol. The binding affinity of drugs, such as vinblastine, vincristine, and paclitaxel, with a molecular weight of between 800 and 900, was hardly affected by cholesterol. From these results, we proposed that the drug-binding site of ABCB1/MDR1 best fits drugs with a molecular weight between 800 and 900, and when small drugs bind to ABCB1/MDR1, cholesterol may fill the empty space. We have demonstrated that the bulkiness of side chains at the position of His61 and its neighboring amino acid residues in TM1 is important for substrate specificity (Taguchi et al. 1997a, b). For example, the replacement of His61 by amino acids with bulkier side chains increased resistance to small drugs such as colchicine and VP16, while it lowered resistance to a large drug, vinblastine. Recently, it was also suggested that TM1 forms part of the drug-binding pocket by cross-linking experiments using the thiol-reactive analog of verapamil (Loo et al. 2006). These observations also suggest that the size of the drug-binding pocket is important for recognizing drugs. We assume that ABCB1/MDR1 and ABCB4/MDR2 have similar substrate binding sites which recognize drugs and cholesterol or PC and cholesterol.

Cholesterol fill-in model for substrate recognition by ABC proteins

Comparative analysis of the functions of various ABC proteins suggests that (1) ABC proteins, either lipid or drug transporters, have similar substrate binding sites which recognize PL and cholesterol, or drugs and cholesterol; (2) Cholesterol in membranes binds to various ABC proteins together with PL or drugs, and plays an important role in substrate recognition, especially by ABCB1/MDR1. Thus, cholesterol fills the empty space in the substrate binding site when small drugs bind to ABCB1/MDR1. We propose calling the mechanism the “cholesterol fill-in model”. ABC proteins exert very flexible substrate recognition, i.e., one-to-many interaction rather than the conventional rigid one-to-one interaction.

As SM is synthesized in the lumen of trans-Golgi, and transferred by vesicular transport, SM mainly exists in the outer leaflet. PC also preferentially exists in the outer leaflet of the plasma membrane. Substrate binding sites of ABCA1 and ABCG1 could exist in the outer leaflet, and lipids could enter the binding site either from the outer leaflet or the inner leaflet of the plasma membrane (Fig. 2). In terms of mechanistic aspects, the conformational change, like the motion in TM α-helix bundles, induced by ATP binding may not cause the substrates to flop but just makes the binding sites hydrophilic, which facilitates the dissociation of substrates from the binding site to the medium or directly to the acceptor. To verify these models, 3D-structural analyses of eukaryote drug-transporting and lipid-transporting ABC proteins are required. The trial of generating 3D crystals of human ABC proteins is in progress.

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