Position 834 in TM6 plays an important role in cholesterol and phosphatidylcholine transport by ABCA1

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ATP-binding cassette protein A1 (ABCA1) plays a key role in eliminating excess cholesterol from peripheral cells by generating nascent high-density lipoprotein (HDL). However, it remains unclear whether both phospholipids and cholesterol are directly loaded onto apolipoprotein A-I (apoA-I) by ABCA1. To identify the amino acid residues of ABCA1 involved in substrate recognition and transport, we applied arginine scan mutagenesis to residues L821–E843 of human ABCA1 and predicted the environment to which each residue is exposed. The relative surface expression of each mutant suggested that residues L821–E843 pass through the plasma membrane as TM6, and the four residues (S826, F830, L834, and V837) of TM6 are exposed to the hydrophilic internal cavity of ABCA1. Furthermore, we showed that L834 is critical for the function of ABCA1.

Keywords: ABC protein; ABCA1; high-density lipoprotein (HDL); cholesterol; transporter

Cholesterol, a key component of cell membranes, is required for cell proliferation; however, excess accumulation of cholesterol is toxic to cells, and excess deposition in peripheral tissues causes atherosclerosis. Therefore, intracellular cholesterol concentration is strictly maintained by various mechanisms, including regulation of synthesis, storage, uptake, and elimination. ATP-binding cassette protein A1 (ABCA1) plays a key role in eliminating excess cholesterol from peripheral cells by generating nascent high-density lipoprotein (HDL), which consists of phosphatidylcholine (PC), cholesterol, and apolipoprotein A-I (apo A-I). Because the defect in ABCA1 causes Tangier disease, in which patients have very low or absent circulating HDL,1–3 it is clear that ABCA1 is essential for HDL generation. However, many questions persist regarding the mechanism of HDL generation, e.g. whether both phospholipids and cholesterol are substrates for ABCA1; and whether lipids are directly loaded onto apo A-I by ABCA1. To address these questions, in this study, we tried to identify the amino acid residues of ABCA1 involved in substrate recognition and transport.

Like ABCA1, MDR3 (also called ABCB4) transports PC as a physiological substrate; it functions in canalicular membrane of hepatocyte and is involved in bile formation.4) Because MDR3 shares a highly conserved amino acid sequence (76% identity and 86% similarity) with MDR1, a multi-drug transporter,5) it is predicted that MDR1 and MDR3 share similar mechanisms of substrate recognition and transport. Indeed, like MDR1, MDR3 also transports various drugs under certain conditions and is inhibited by cyclosporine A and verapamil, inhibitors of MDR1.6–8) Previously, we reported that cyclosporine A and its non-immunosuppressive analog, PSC833, also inhibit ABCA1 via direct binding,9) suggesting that MDR1, MDR3, and ABCA1 share similar substrate-binding sites. TM6 of MDR1 plays an important role in substrate recognition;10,11) hence, we predicted that TM6 of ABCA1 is also involved in substrate recognition. Recently, we determined the three-dimensional (3D) structure of CmABCB1, an MDR1 ortholog of Cyanidioschyzon merolae, at 2.4 Å resolution.12) The structure contains a spacious internal cavity, in which the substrate-binding site is predicted to be located, and a portion of TM6 faces the internal cavity. Therefore, we predicted that a portion of TM6 of ABCA1 would also face the internal cavity and be involved in substrate recognition.

To test this prediction, we first assigned the orientation of TM6 of ABCA1 by replacing each amino acid residue in TM6 with an arginine to investigate whetherarginine
can be accommodated at each position. Because arginine has a large side chain with positive charges, the introduction of this residue to positions that interact with the lipid bilayer or other TMs should disrupt the protein folding of ABCA1, hindering protein trafficking from the ER to the plasma membrane. By contrast, if an arginine residue is introduced to a position facing the internal cavity, it would not affect protein folding or trafficking to the plasma membrane. After determining which residues were predicted to face the internal cavity, we then analyzed their functions.

**Materials and methods**

**Plasmids and transfection.** The expression vectors for wild-type ABCA1, ABCA1-K939 M, and K1952 M (ABCA1MM), in which two lysine residues (K939 and K1952) crucial for ATP hydrolysis were replaced by methionine and all the ABCA1 mutants were constructed by using In-Fusion Kit (Takara Bio). The integrity of the mutated DNA was confirmed by sequencing. ABCA1 cDNA was fused with the green fluorescent protein (GFP) at the C-terminus and then inserted into the plRESPuro3 vector (Takara Bio). The influenza virus hemagglutinin (HA) epitope sequence (coding YPYDVPDYA) was introduced between G207 and D208 as previously reported. Human embryonic kidney (HEK) 293 cells were transfected with each expression vector using Lipofectamine LTX with Plus Reagent (Invitrogen). Stable transformants were selected in the presence of 1 μg/mL puromycin.

**Anti-HA antibody immunostaining.** Cells grown on collagen-coated coverslips were fixed with 4% paraformaldehyde at room temperature for 30 min. After blocking with PBS(+) containing 1% BSA, the cells were incubated with anti-HA antibody (1 μg/ml) in PBS(+) containing 0.02% BSA for 15 min at room temperature. After washing with PBS(+), cells were incubated with an Alexa Fluor 546-conjugated secondary antibody and observed on a confocal microscope. To compare the surface expression of ABCA1, the intensity of Alexa Fluor 546 of 10 cells was analyzed with ImageJ and normalized to the GFP intensity.

**Flow cytometry analysis.** HEK293 cells were harvested after trypsinization and washed twice with Hanks’ balanced salt solution (HBSS). The cells were then incubated with both anti-HA (F-7) antibody (Santa Cruz Biotechnology) (1/200 diluted) and an Alexa Fluor 633-conjugated secondary antibody (1/500 diluted) at room temperature for 30 min and analyzed with a flow cytometer (Accuri C6, BD). The amount of ABCA1 on the cell surface was calculated from the histogram of double-positive (GFP+HA+) cells.

**Cellular lipid release assay.** Cells were incubated in the presence of 10 μg/mL apolipoprotein A-I (apoA-I) for 24 h in Dulbecco’s modified Eagle’s medium (DMEM) containing 0.02% bovine serum albumin (BSA). The cholesterol and PC contents in the medium were determined using a colorimetric enzyme assay or a fluorescence enzyme assay.

**Statistical analysis.** Values are presented as the means ± SD (n ≥ 3). Statistical significance was determined by Dunnett’s test.

**Results**

Single arginine mutations were introduced at positions 821–843 of human ABCA1, which, based on predictions by SOSUI and PredictProtein, likely spans the membrane as TM6. HEK293 cells were transfected with the mutant cDNAs, and cell surface localization was monitored by immunostaining of the HA tag inserted at the position of 207 in the extracellular loop (Fig. 1(A)). This peptide insertion had no effect on the subcellular localization or the function of ABCA1. The relative surface expression of each mutant was calculated by dividing HA immunofluorescence by GFP fluorescence, and was compared with that of wild-type ABCA1 (Fig. 1(B)). Mutants could be classified into two groups: group 1 mutants were localized to the plasma membrane at more than ~50% of the wild-type efficiency, and group 2 mutants were localized to the plasma membrane at less than 25% of the wild-type efficiency. Group 1 (filled bars) consisted of 14 mutants: L821R, T822R, T823R, S826R, S828R, F830R, L834R, G836R, V837R, T839R, W840R, Y841R, I842R, and E843R. Group 2 (empty bars) consisted of nine mutants: V825R, M827R, M828R, L829R, D831R, T832R, F833R, Y835R, and M838R.

Amino acid residues of group 1 (filled circles) and group 2 (empty circles) were placed in the helix model, and two faces (A and B) of the helix are shown in Fig. 2. This model suggested three features of TM6: (i) arginine replacement of residues predicted to be located at either end of the helix (L821, T822, T823, S826, G836, V837, T839, W840, Y841, I842, and E843) did not affect protein trafficking, suggesting that these residues are in a hydrophilic environment; (ii) all the residues whose replacement severely affected protein trafficking (V825, M827, M828, L829, D831, T832, F833, Y835, and M838) are located in the middle of the helix, suggesting that they face the hydrophobic environment of membrane lipids or are involved in helix-helix interactions; and (iii) four amino acid residues (S826, F830, L834, and V837) whose replacement did not affect protein trafficking formed a line along the B face from the extracellular side to the cytosolic side (Fig. 2). The first two features are consistent with the prediction that the amino acid residues from L821 to E843 pass through the membrane as TM6. The third feature suggested that the four positions (S826, F830, L834, and V837) face the hydrophilic internal cavity of the protein, which can accommodate the large hydrophilic side chain of arginine.

**Lipid export activity of arginine mutants**

Next, to determine whether the four amino acid residues that face the internal cavity (S826, F830, L834, and
V837) are involved in substrate recognition or the transport process of ABCA1, we established cells stably expressing the respective arginine mutants. Because W840, whose arginine substitution was found in a Tangier disease patient, was mapped one turn below of V837, we also established cells stably expressing the V840R mutant. Cells stably expressing the D831R mutant, which should not be localized to the plasma membrane, were established as a negative control. We used flow cytometry analysis with an antibody against HA peptide to determine how efficiently each ABCA1 variant was localized to the cell surface (Fig. 3(A)). In the case of cells expressing wild-type ABCA1(207HA)-GFP or five mutants (S826R, F830R, L834R, V837R, and W840R), the HA and GFP fluorescence intensities were well correlated, suggesting that these five mutant ABCA1 proteins were localized to the plasma membrane as efficiently as the wild type. In the case of D831R mutant, however, the HA and GFP fluorescence intensities were not correlated, suggesting that this mutation hindered trafficking to the plasma membrane, as predicted in Fig. 1. Next, we measured apo A-I-dependent

![Fig. 1. Expression of the ABCA1-GFP mutant on the plasma membrane.](image)
cholesterol and PC efflux from these mutants (Supplemental Fig. 1) and normalized lipid efflux efficiency to the total amount of ABCA1 on the cell surface, as described in Materials and Methods section (Fig. 3(B)). The lipid efflux efficiency of L834R mutant was as low as that of the non-functional mutant ABCA1MM, whereas the S826R, F830R, and V837R mutants exhibited cholesterol and PC efflux activity of as high as that of the wild type (Fig. 3(B)). The W840R substitution, which was found in a Tangier disease patient, did not affect the function of ABCA1 (Fig. 3(C)). These results suggest that among the five amino acid residues predicted to be lined up along the B face of TM6 (Fig. 2), only L834R affects the function of ABCA1, although it does not affect protein folding.

Effect of amino acid substitution of L834 on lipid export activity

The results described above suggested that L834 is critical for substrate recognition or transport by ABCA1. To elucidate the role of L834 on the function of ABCA1, we substituted L834 with the 18 remaining amino acid residues (i.e. other than L and R). Cells stably expressing each mutant were established, and cell surface expression was analyzed. HA and GFP immunofluorescence intensities were well correlated in cells expressing most mutants, with the exceptions of L834D, L834E, and L834Q (Supplemental Fig. 2). We found that L834D, L834E, and L834Q could be localized to the cell surface when they were transiently transfected (Supplemental Fig. 3). Lipid efflux from L834 mutants was measured and normalized to the total of ABCA1 on the cell surface (Fig. 4). The results revealed that the presence of 17 amino acid residues did not significantly affect lipid efflux activity of ABCA1 or the ratio of cholesterol efflux to PC efflux, whereas substitution with lysine and arginine reduced and abolished the lipid efflux activity (both cholesterol and PC efflux) of ABCA1, respectively.

Discussion

In this study, we applied arginine scan mutagenesis to amino acid residues L821–E843 of human ABCA1 to predict the environment to which each residue of TM6 is exposed. Based on the output of SOSUI and PredicProtein, these residues are expected to form TM6. The relative surface expression of each mutant suggested that both ends of the helix, formed by L821–E843, are in a hydrophilic environment, whereas the middle part is in a hydrophobic environment. Thus, as expected, amino acid residues L821–E843 pass thorough the plasma membrane as TM6.

Arginine scan mutagenesis was first performed by Loo et al.19). Those authors introduced single arginine mutations to a processing mutant (G251V) of MDR1 (ABCB1), which is defective in folding and trafficking to the cell surface, and succeeded in identifying amino
acid residues whose arginine mutations promoted maturation. The results suggested that those residues faced an aqueous drug translocation channel within MDR1. In this study, we applied arginine scan mutagenesis to wild-type ABCA1 and succeeded in predicting the environment that each residue faces, suggesting that this method is also effective when applied to the wild-type protein.

The results of arginine scan mutagenesis suggested that the four amino acid residues (S826, F830, L834, and V837), whose replacement did not affect the protein trafficking, lined up along one face of TM6 from the extracellular side to the cytosolic side of the membrane (Fig. 2). Recently, we determined the 3D structure of CmABCB1, an MDR1 ortholog of Cyanidioschyzon merolae, at 2.4 Å resolution. The structure contains a spacious internal cavity, in which the substrate-binding site is predicted to be located, and a portion of TM6 faces the internal cavity. Face A of TM6 of ABCA1 contains four amino acid residues (S826, F830, L834, and V837) that are predicted to be exposed to the hydrophilic internal cavity, which can accommodate the large hydrophilic side chain of arginine.

The amino acid substitution of L834 revealed that only arginine and lysine affected the lipid transport activity of ABCA1. Arginine abolished transport almost completely, whereas lysine had a more moderate effect. This observation suggested that the function of ABCA1 is affected by the length of the side chain, the number of positive charges, or both of L834. However, the ratio of transported cholesterol and PC was unchanged. Negative charges of the side chain did not affect its function, whereas it slightly affected trafficking to the plasma membrane. These results suggest that the side chain of position 834 is not directly involved in substrate recognition. Alternatively, position 834 could play an important role in conformational changes during the transport process. The L834R mutant may make the inward-open form quite stable and hinder the conformational change to the outward-open form. Indeed, apo A-I did not bind to the ECD of the L834R mutant (data not shown), which is believed to be dependent on the conformational change of ABCA1 after ATP hydrolysis. The amino acid residues of TM6 including L834 are conserved among mammalian ABCA1 proteins, also indicating the importance of this transmembrane helix for the functions of ABCA1.

Probost et al. reported that a Tangier patient was heterozygous for two mutations, which result in the amino acid substitutions W840R and N935S. N935, which is in the highly conserved Walker A motif of the
amino-terminal ATP-binding domain, is a Tangier mutation originally reported by Bodzioch et al.1). Because family members that are heterozygous for N935S showed subnormal plasma HDL levels but have mild disease,1) and because a patient with heterozygous for W840R and N935S showed severe HDL deficiency, it was predicted that W840R is a Tangier mutation.18) However, it was not clear whether W840R substitution itself affects the function of ABCA1. In this study, we observed no obvious effects of this mutation on the trafficking and function of ABCA1 when expressed in HEK293 cells, suggesting that this mutation does not impair the transport activity of ABCA1 when expressed on the cell surface. Thus, W840R might affect protein trafficking or some type of post-translational regulation of ABCA1 in vivo.

In summary, based on our arginine scan mutagenesis, we predicted that four amino acid residues (S826, F830, L834, and V837) of TM6 are exposed to the hydrophilic internal cavity of ABCA1, and identified L834 as critical for the function of ABCA1. These findings should facilitate the study of the functional mechanism of ABCA1 in nascent HDL formation.

**Supplemental material**

The supplemental material for this paper is available at http://dx.doi.org/10.1080/09168451.2014.993358.

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