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Purification and ATPase Activity of Human ABCA1*[S]

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Kei Takahashi, Yasuhisa Kimura, Noriyuki Kioka, Michinori Matsuo, and Kazumitsu Ueda[1]

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

ATP-binding cassette protein A1 (ABCA1) plays a major role in cholesterol homeostasis and high density lipoprotein metabolism. Apolipoprotein A-I binds to ABCA1 and cellular cholesterol and phospholipids, mainly phosphatidylcholine, are loaded onto apoA-I to form pre-β high density lipoprotein (HDL). It is proposed that ABCA1 translocates phospholipids and cholesterol directly or indirectly to form pre-β HDL. To explore the mechanism of ABCA1-mediated pre-β HDL formation, we expressed human ABCA1 in insect Sf9 cells and purified it. Trypsin limited-digestion of purified ABCA1 in the detergent-soluble form suggested that it retained conformation similar to ABCA1 expressed in the membranes of human fibroblast WI-38 cells. Purified ABCA1 showed robust ATPase activity when reconstituted in liposomes made of synthetic phosphatidylcholine. ABCA1 showed lower ATPase activity when reconstituted in liposomes containing phosphatidylserine, phosphatidylethanolamine, or phosphatidylglycerol and also showed weak specificity in acyl chain species. ATPase activity was reduced by the addition of cholesterol and decreased by 25% in the presence of 20% cholesterol. β-Sitosterol and campesterol showed similar inhibitory effects but stigmasterol did not, suggesting structure-specific interaction between ABCA1 and sterols. Glibenclamide suppressed ABCA1 ATPase, suggesting that it inhibits apoA-I-dependent cellular cholesterol efflux by suppressing ABCA1 ATPase activity. These results suggest that the ATPase activity of ABCA1 is stimulated preferentially by phospholipids with choline head groups, phosphatidylcholine and sphingomyelin. This study with purified human ABCA1 provides the first biochemical basis of the mechanism for HDL formation mediated by ABCA1.

ATP-binding cassette protein A1 (ABCA1)[2] plays a major role in cholesterol homeostasis and high density lipoprotein (HDL) metabolism. It has been reported that apolipoprotein A-I (apoA-I) binds to ABCA1 and cellular free cholesterol (FC) and phospholipids (PL) are loaded onto apoA-I to form pre-β HDL. It is clear that ABCA1 is involved in phosphatidylcholine (PC)-rich HDL generation in plasma, because plasma PL concentration of *Abca1*−/− mice was decreased by more than 75%, mostly due to a reduction of PC in HDL (1); however, the molecular mechanism behind ABCA1-mediated pre-βHDL formation is still poorly understood.

Several models have been proposed for the mechanism of ABCA1-mediated pre-β HDL formation: (*a*) a two-step process model proposed by Fielding *et al.* (2) and Wang *et al.* (3): ABCA1 first mediates PL efflux to apoA-I, and this apolipoprotein-PL complex accepts FC in an ABCA1-independent manner. This model is based on two types of experiments: (i) vanadate, glibenclamide, and cyclodextrin show differential inhibitory effects upon PL and FC efflux to apoA-I, and (ii) medium containing apoA-I conditioned on smooth muscle cells leads to FC efflux from vascular endothelial cells that do not express ABCA1. (*b*) A concurrent process model: FC and PL efflux by ABCA1 to apoA-I are tightly coupled to each other (4). (*c*) PS flipping model: ABCA1 mediates the translocation of 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 (5).

ABC proteins involved in xenobiotic efflux, such as MDR1 and MRP1, harness the energy liberated from ATP to drive the conformation changes that move xenobiotics across the membrane, and mutations in the ATP binding domain abolish the transport activity of these proteins (6, 7). Like these xenobiotic transporters, ABCA1 K939M mutant, in which lysine, indispensable for the hydrolysis of ATP by various ABC proteins (8–11), was substituted by methionine, was impaired in apoA-I-dependent PL and cholesterol efflux (3, 5). As the translocation (flip-flop) of PLs rarely spontaneously occurs in lipid bilayers, and this process is highly energy-dependent, ABCA1 is suggested to flip-flop PLs depending on ATP hydrolysis. However, because the ABCA1 K939M mutant is defective in its interaction with apoA-I (3, 5), it is also possible that ATP binding and/or ATP hydrolysis cause conformational changes of ABCA1, which are required for the interaction with apoA-I, and ABCA1 functions as a regulator in HDL formation (12) as SUR does in the ATP-sensitive potassium channel complex (13).

Human ABCA7, which has the highest homology (66.1%) to ABCA1, mediates the apoA-I-dependent efflux of phospholipids and cholesterol as ABCA1 (14); however, human ABCA7 mediates cholesterol release with much less efficiency than ABCA1 (15), and it has been reported that phospholipids but not cholesterol are loaded onto apoA-I by mouse ABCA7 (16). These results may not be explained by the two-step process model and suggest that ABCA1 and ABCA7 have different substrate specificities for transport and that cholesterol is one of the transport substrates for ABCA1.

To explore the mechanism of ABCA1-mediated pre-β HDL formation, we purified human ABCA1 as a detergent-soluble form and examined ATPase activity. Purified ABCA1 showed robust ATPase activity when reconstituted in liposomes made of synthetic PC, and the ATPase

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–4.

[1] To whom correspondence should be addressed. Tel.: 81-75-753-6124; Fax: 81-75-753-6104; E-mail: uedak@kais.kyoto-u.ac.jp.

[2] The abbreviations used are: ABCA1, ATP-binding cassette protein A1; ABCA1 MM, ABCA1 K939M-K1952M; MDR, multidrug resistance; MRP, multidrug resistance-related protein; NBF, nucleotide-binding fold; apo, apolipoprotein; HDL, high density lipoprotein; FC, free cholesterol; PL, phospholipid; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; SM, sphingomyelin; PG, phosphatidylglycerol; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; DPPC, 1,2-dipalmitoylphosphatidylcholine; POPS, 1-palmitoyl-2-oleoylphosphatidylserine; DPPE, 1,2-dipalmitoylphosphatidylethanolamine; DPPG, 1,2-dipalmitoylphosphatidylglycerol; Sf9, *Spodoptera frugiperda* 9; HEK, human embryo kidney; NGF, *N*-glycosidase F; DDM, *n*-dodecyl-β-D-maltoside; NTA, nitrilotriacetic acid.

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activity was inhibited by glibenclamide but not by vanadate. ABCA1 ATPase was reduced by the addition of cholesterol.

EXPERIMENTAL PROCEDURES

Materials—Bovine serum albumin, leupeptin, aprotinin, trypsin, glibenclamide, stigmasterol, campesterol, and Na_2ATP were purchased from Sigma. Baculovirus transfer vector (pVL1392) and baculovirus (Baculogold™) were obtained from Invitrogen. Anti-penta-His antibody was obtained from Qiagen. L-α-Lecitin (20%) from soybean and sphingomyelin from egg yolk were obtained from Sigma. Synthesized phospholipids, PC, phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cholesterol were purchased from Avanti polar lipids. β-Sitosterol and brassicasterol were purchased from Tama Biochemical Co., Ltd. Other chemicals were purchased from Wako Pure Chemical Industries Ltd. KM3073 monoclonal antibody was generated against the first extracellular domain (35–635 amino acid) of human ABCA1 in rats as described previously (17). Anti-ABCA1 NBF2 rabbit polyclonal antibody, which specifically interacts with the second nucleotide binding fold (NBF2) (data not shown), was generated against the purified NBF2 of human ABCA1 (1908–2159 amino acids). Anti-ABCA1 C terminus rabbit antibody was a kindly gift from Dr. Shinji Yokoyama, Nagoya City University Graduate School of Medical Sciences.

Construction of Transfer Vector—The 3′ end of human ABCA1 cDNA (18) was modified by elimination of the natural termination codon and insertion of a supplementary sequence containing a thrombin cleavage site, a biotinylation tag, and 10 consecutive histidine residues before the termination codon (5′-CTAGACTGGTTCCGCGTGGTTCCGGCTTGAATGATATATTCGAGGCCCAGAAGATAGAGTGGCATGAGGGAACTAGACTGGTTCCGCGTGGTTCCCACCATCACCATCACCATCACCATCACCATTGAG-3′). The modified ABCA1 cDNA (designated ABCA1-TATH) was inserted into the transfer vector pVL1392. To construct the NBF1 Walker A lysine mutant, ABCA1 K939M, DNA fragments containing a K939M missense mutation were generated by a two-step PCR method with two pairs of primers, 5′-GGGCCACAATGGAGCGGGGATGACGACCAC-3′ and 5′-CTGTCCCCCAGGACGTCCGCTTCATCCATG-3′ and 5′-GTGGTCGTCATCCCCGCTCCATTGTGGCCC-3′ and 5′-CTCAGTGGCTGTGATCATCAAGGGCATCG-3′. NBF2 Walker A lysine-1952 was substituted with methionine using a QuikChange site-directed mutagenesis kit (Stratagene) with a mutagenic primer, 5′-GGGGCTGGAATGTCATCAACTTTC-3′. The hMDR1 expression vector and recombinant virus were generated as described previously (6).

Generation of Recombinant Baculovirus—Recombinant baculovirus containing ABCA1-TATH (BV-ABCA1-TATH) was generated by the co-transfection of *Spodoptera frugiperda* 9 (Sf9) cells with pVL1392-ABCA1-TATH and Baculogold™ DNA. BV-ABCA1-TATH was purified and amplified following the manufacturer's directions. The generated virus was kept at 4 °C in the dark.

Expression of Human ABCA1 in Sf9 Cells—Sf9 cells were grown at 27 °C as a monolayer culture in Grace's insect medium (Invitrogen) with 10% fetal bovine serum or as a suspension culture in Grace's insect medium with 10% fetal bovine serum plus 0.1% purulonic F-68 (Invitrogen). Sf9 cells were infected with BV-hABCA1-TATH at a multiplicity of infection of 5. At 48 h after infection, cells were harvested and washed with ice-cold phosphate-buffered saline. Cells were stored at −80 °C.

Preparation of Microsomal Membrane—All the steps in the preparation of the microsomal membrane fraction were performed at 0–4 °C. Sf9 cells were thawed and resuspended in 10×cell volume of sonication buffer containing 20 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, 1 mM EDTA, and protease inhibitor mixture (100 μg/ml (*p*-amidinophenyl)-methanesulfonyl fluoride, 10 μg/ml leupeptin, and 2 μg/ml aprotinin). The cell suspension was sonicated for 5 min (output 5, 10 rounds of sonication for 30 s + interval of 2 min) with a probe tip-type sonicator (Misonix Inc.) and centrifuged at 3,000 × *g* for 10 min to remove unbroken cells and nuclei. The supernatant was centrifuged at 40,000 × *g* for 60 min. The microsome pellet was resuspended in ice-cold buffer A containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 30% (v/v) glycerol, 10 mM imidazole, 1 mM 2-mercaptoethanol, and protease inhibitor mix. The microsomal membrane suspension was passed through a 22-gauge syringe five times and stored at −80 °C. Micorosomal membrane protein (80–100 mg) was obtained from 10 g of wet cells.

Glycosylation Analysis—The reaction of *N*-glycosidase F (NGF) was performed as described previously (18). Lectin staining was performed as follows: After SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane. The membrane was first blocked with block ACE (Dainihon Pharmaceutical) and incubated with 1 μg/ml of concanavalin A (Honen Corp.) in buffer containing 0.15 M NaCl, 0.01 M Tris-HCl (pH 7.5), and 0.05% Tween 20. The polyvinylidene difluoride membrane was then washed three times, and signals were detected with an ECL detection kit (Amersham Biosciences).

Extraction of ABCA1 from Sf9 Membrane—The microsomal membrane was resuspended with buffer A containing 0.6 or 0.8% *n*-dodecyl-β-D-maltoside (DDM) (Dojindo) and protease inhibitor mix and kept on ice with occasional gentle mixing for 30 min. The insoluble fraction was removed by centrifugation (100,000 × *g*, 60 min) in a TLA 100.4 rotor (Beckman).

Purification with Ni^{2+}-NTA-Agarose—All purification steps were performed at 0–4 °C. Extracted proteins were applied to Ni^{2+}-NTA-agarose (Qiagen) pre-equilibrated with buffer A. The mixture was rotated for 18 h. The resin was then washed with 20× bed volume of buffer A containing 0.1% DDM and 20 mM imidazole. ABCA1 or MDR1 was eluted with 2× bed volume of buffer A containing 0.1% DDM and 200 mM imidazole. The eluate was concentrated by ultrafiltration using a microcon YM-100 (Millipore) to 0.1–0.3 mg/ml protein. Protein concentrations were determined by the Bradford method using a protein assay kit (Bio-Rad) (19). Bovine serum albumin was used as a standard.

Purification with Anion Exchange Chromatography Sepharose, DE52—Proteins were mixed with pre-equilibrated DE52 Sepharose (Whatman) in DE52 binding buffer (50 mM Tris-HCl (pH 7.4), 30%(v/v) glycerol, 0.1% DDM, 1 mM 2-mercaptoethanol), and the mixture was rotated for 12 h. ABCA1 was eluted with DE52 binding buffer containing NaCl (50∼200 mM) for 60 min. The flow-through fraction and eluate were concentrated by ultrafiltration using a microcon YM-100 to 0.1–0.2 mg/ml protein.

Cell Culture and Membrane Preparation—WI-38 human fibroblast cells and HEK293 stably expressing human ABCA1, generated by hygromycin selection as described previously (17), were grown at 37 °C in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. WI-38 cells were seeded into a 100-mm dish at 5×10^6 cells. To induce ABCA1 expression, cells were cultured in the medium containing 10 μM TO901317 and 5 μM 9-*cis*-retinoic acid for 24 h. Crude membranes were prepared by nitrogen cavitation method as described previously (18).

Reconstitution of Purified ABCA1 with Lipids—L-α-Lecitin (20%) from soybean, sphingomyelin, or synthesized phospholipids dissolved in chloroform were dried by evaporation and resuspended in reaction buffer, 40 mM Tris-HCl (pH 7.5), 0.1 mM EGTA to a final concentration of 5 mg/ml. When sterols were incorporated into the liposomes, sterols were mixed with phospholipids in chloroform at first, then dried by

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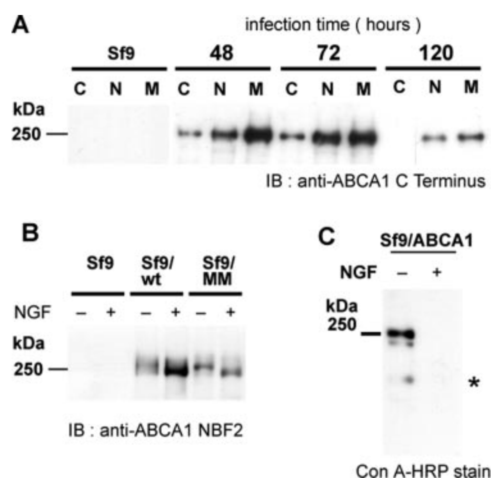

FIGURE 1. **Membrane localization and glycosylation of human ABCA1 expressed in Sf9 cells.** *A*, Western blotting of human ABCA1-TATH protein expressed in Sf9 cells. Proteins in cytosolic (*C*), nuclear (*N*), and microsomal (*M*) fractions (10 μg) were separated on 7% SDS-PAGE gel. ABCA1-TATH was detected with anti-ABCA1 terminus antibody. *B*, membranes prepared from Sf9 control cells or cells transfected with ABCA1-TATH, wild-type (*wt*), and mutant (MM) virus were treated with or without NGF. Proteins were separated on 7% SDS gel, and ABCA1-TATH was detected with anti-penta-His antibody. *C*, membranes prepared from Sf9 cells transfected with ABCA1-TATH virus were treated with or without NGF. Glycoproteins were detected with concanavalin A-conjugated horseradish peroxidase. A band marked by an *asterisk*, was also detected in Sf9 control cells (data not shown).

evaporation and resuspended in reaction buffer according to the standard procedure (20). The suspension was sonicated in a bath sonicator. To examine the effect of apoA-I, apoA-I (500 ng) was added to the suspension either before or after sonication. The lipid stock was stored at 4 °C under N_2 gas and kept in a dark place. Purified protein (100 ng) was mixed with 500 ng of lipid, and the mixture was incubated at 23 °C for 20 min. The reconstitution buffer for MDR1 contained 2 mM dithiothreitol.

Assay of ATPase Activity—Reactions were carried out in 16 μl of 40 mM Tris-HCl (pH 7.5), 0.1 mM EGTA, 10 mM Na_2ATP (pH 7.0), and 10 mM $MgCl_2$ at 37 °C for 30 min. Reactions were initiated by the addition of purified reconstituted ABCA1 (100 ng) and stopped by the addition of 14 μl of 12% SDS and vigorous mixing. ATPase activity was analyzed using two methods: (i) measuring released ADP by high performance liquid chromatography with titanium dioxide (21) and (ii) measuring released P_i by the P_i-Mo method (22, 23). The P_i released by the reaction with reconstituted ABCA1, predenatured by SDS, was subtracted as a negative control. Little or no ATP hydrolysis was observed in the reaction without protein.

RESULTS

Expression of Human ABCA1 in Insect Cells—We constructed a plasmid, pVL1392-ABCA1-TATH, to express human ABCA1 fused with a histidine tag and biotinylation tag at the C terminus, and this was introduced into insect Sf9 cells. Individual plaques were examined for the expression, and Sf9 cells were infected by the purified virus for large scale production and purification. Human ABCA1 expressed in Sf9 cells, about 250 kDa, was found most abundantly in the microsome membrane fraction in 48 h (Fig. 1*A*), and ABCA1 in the nuclear fraction increased in 72 h. The amount of ABCA1 decreased in 120 h probably due to degradation. Therefore, Sf9 cells were harvested at 48–72 h after infection. The amount of ABCA1 in the microsome membrane fraction accounted for about 1% of total membrane proteins.

Glycosylation of ABCA1 Expressed in the Sf9 Membrane—ABCA1 is glycosylated and has two large extracellular domains, which are suggested to be functionally important (24, 25). The glycosylation of ABCA1 expressed in Sf9 membranes was examined. The mobility of ABCA1, expressed in Sf9 membranes, in SDS-PAGE became faster by NGF treatment (Fig. 1, *B* and *C*). ABCA1 reacted with concanavalin A, which reacts mainly with high mannose-type sugar chains, but ABCA1 treated with NGF did not (Fig. 1*C*). These results suggest that human ABCA1 expressed in Sf9 is modified with high mannose-type sugar chains.

Solubility and Purification of ABCA1—As nonionic-detergent DDM was successfully used to solubilize recombinant ABC proteins (9, 26, 27), human ABCA1 expressed in the Sf9 membrane was extracted with 0.6% and 0.8% DDM (Fig. 2*A*). ABCA1, extracted with 0.6% and 0.8% DDM (*lanes 1* and *3*), migrated a little slower than it remained in the insoluble fraction (*lanes 2* and *4*). Roughly 50% of ABCA1 was extracted with DDM, and efficiency was not significantly increased even with 1.0% DDM (data not shown).

Extracted ABCA1 was purified using Ni^{2+}-NTA-agarose resin (Fig. 2*B*). ABCA1 was recovered from the resin with 200 mM imidazole (*lanes 10* and *11*) in 80–90% purity judged from Coomassie Brilliant Blue staining. Purity was not significantly different between 0.6 and 0.8% DDM extraction. About 100 μg of purified ABCA1 was obtained from 250 mg of microsome membranes of Sf9 cells cultured in 1L suspension culture.

Initially we planned to further purify ABCA1 with avidine column after *in vitro* biotinylation of the tag fused at the C terminus as reported previously (28); however, ABCA1 was not successfully recovered from the avidine column (data not shown). Therefore, we performed anion exchange chromatography using DE52 Sepharose (Fig. 2*C*). ABCA1, recovered from Ni^{2+}-NTA-agarose resin, was concentrated and mixed with pre-equilibrated DE52 Sepharose. The majority of ABCA1 did not bind to DE52 Sepharose, and ABCA1 could be purified as a flow-through fraction of DE52 (Fig. 2*C*). ABCA1 was recovered in almost pure form from DE52 Sepharose judging from Coomassie Brilliant Blue (Fig. 2*C*) and silver staining (Fig. 2*D*). ABCA1 K939M-K1952M protein, expressed and purified in the same procedure, was as pure as the wild type (Figs. 1*B* and 2*E*).

Trypsin Sensitivity of ABCA1—To confirm that purified ABCA1 retained the correct conformation after the purification procedure, we examined the trypsin sensitivity of ABCA1. ABCA1, endogenously expressed in human fibroblast WI-38 cells, was partially digested by trypsin. ABCA1 was partially cleaved to produce four fragments, 170 and 150 kDa and subsequently 125 and 110 kDa, which were recognized by anti-ABCA1 extracellular domain 1 antibody (KM3073) (Fig. 3*A*) and two fragments, 170 and 120 kDa, which were recognized by anti-ABCA1 NBF2 antibody (Fig. 3*B*). These fragments may be assigned to fragments digested at site A, just after the sixth transmembrane α-helix, and at site B, just before the seventh transmembrane α-helix as shown in Fig. 3*F*. It is predicted that the limited digestion at site A produces 150- and 110-kDa fragments and their glycosylated forms, and the limited digestion at site B produces 155- and 110-kDa fragments and their glycosylated forms as a diagram (Fig. 3*F*). When these fragments were analyzed by SDS-PAGE under non-reducing conditions, they co-migrated with undigested ABCA1 of about 280 kDa (supplemental Fig. 2). These results suggested that N and C halves of ABCA1 were connected with disulfide bonds.

When ABCA1 expressed in Sf9 cells was partially digested with trypsin, ABCA1 produced 140-kDa and subsequently 95-kDa fragments, which were recognized by KM3073 (Fig. 3*C*), and 155-kDa and subsequently 110-kDa fragments, which were recognized by anti-ABCA1 NBF2 antibody (Fig. 3*D*). All the produced fragments were smaller than

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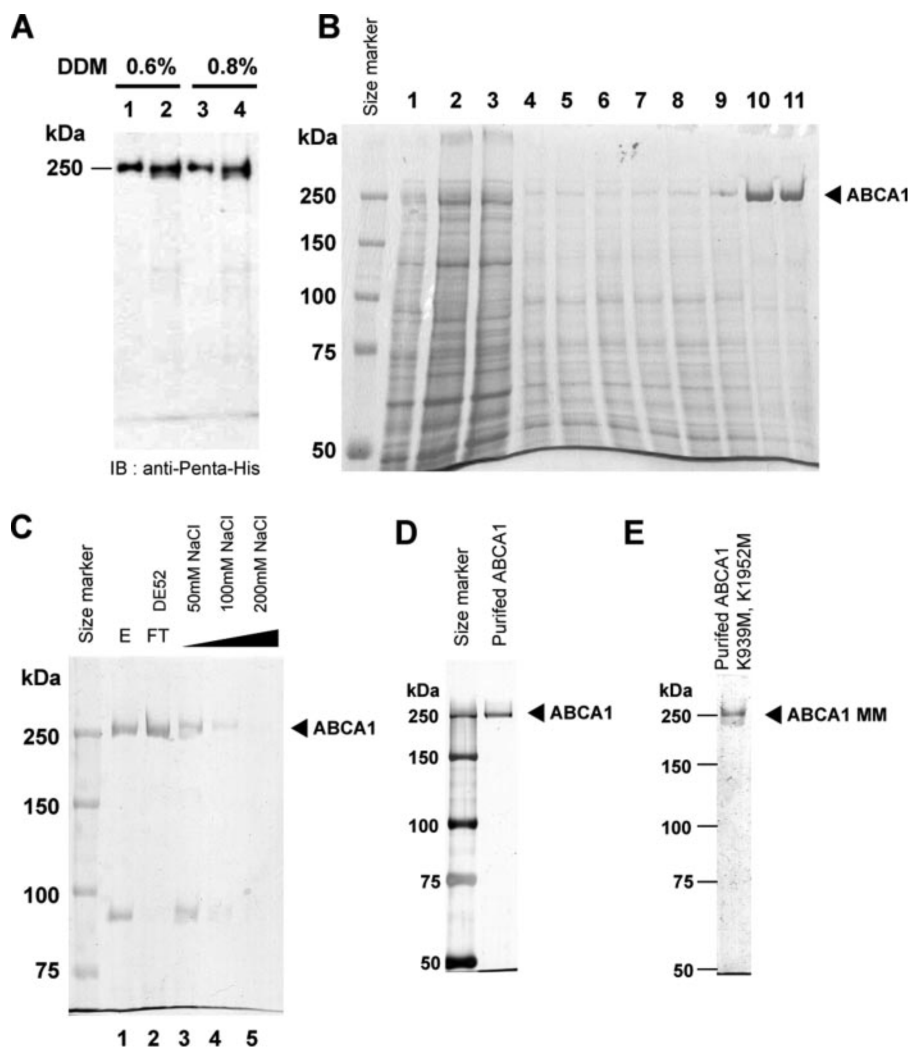

FIGURE 2. **Purification of ABCA1.** *A*, Sf9 membranes were extracted with 0.6% (*lanes 1* and *2*) and 0.8% DDM (*lanes 3* and *4*). *Lanes 1* and *3*, soluble fraction; *lanes 2* and *4*, insoluble fraction. Proteins (10 μg) were separated on 7% polyacrylamide gel, and ABCA1-TATH was detected with anti-penta-His antibody. *B*, Coomassie Brilliant Blue R-250 staining: *lane 1*, microsomes 10 μg; *lanes 2* and *3*, insoluble fraction after DDM extraction of microsomes 10 μg; *lanes 4* and *5*, soluble fraction after DDM extraction of microsomes 5 μg; *lanes 6* and *7*, flow-through from Ni^{2+}-NTA column, 5 μg; *lanes 8* and *9*, 20 mM imidazole eluate from Ni^{2+}-NTA column, 3 μg; *lanes 10* and *11*, eluate from Ni^{2+}-NTA column with 200 mM imidazole, 3 μg. *C*, Purification by DE52 column (Coomassie Brilliant Blue R-250 staining): *lane 1*, Ni^{2+}-NTA eluate; *lane 2*, DE52 flow-through fraction; *lane 3*, eluate from DE52 with DE52 binding buffer containing 50 mM NaCl; *lane 4*, eluate with buffer containing 100 mM NaCl; *lane 5*, eluate with buffer containing 200 mM NaCl. *D*, silver staining of DE52 flow-through fraction (25 ng). *E*, purified ABCA1 K939M-K1952M mutant, 0.5 μg (Coomassie Brilliant Blue R-250 staining).

the corresponding ones produced from ABCA1 of WI-38 cells probably due to glycosylation differences. When these fragments were analyzed by SDS-PAGE under non-reducing conditions, they co-migrated with undigested ABCA1 of about 250 kDa (supplemental Fig. 2). These results suggested that ABCA1 expressed in WI-38 cells and Sf9 cells contained similar typsin-sensitive sites (Fig. 3*E*).

Trypsin limited-digestion of purified detergent-soluble ABCA1, producing N-terminal 140- and 95-kDa fragments and C-terminal 155- and 110-kDa fragments (Fig. 3*E*). These fragments well corresponded to the fragments produced from ABCA1 endogenously expressed in WI-38 (Fig. 3, *A* and *B*), ABCA1 exogenously expressed in Sf9 cells (Fig. 3, *C* and *D*), and HEK293 cells (supplemental Fig. 1). These results suggest that purified ABCA1 retains conformation similar to ABCA1 endogenously expressed in human fibroblast membranes.

ATPase Activity of ABCA1—The ATPase activity of purified ABCA1 reconstituted in soybean lipids was examined. ATP was hydrolyzed by proteoliposomes in a time-dependent manner at 37 °C, and released phosphate ions increased linearly during 30 min (Fig. 4). Hence, the following ATPase assays were performed at 37 °C for 30 min in this study.

Next, we examined ATPase activity in the presence of various concentrations of MgATP. ATPase increased with increasing concentrations of ATP, and the apparent K_m for ATP and V_{max} were 112 μM and 455 nmol/min/mg of protein, respectively. The ATPase activity of purified reconstituted ABCA1 varied ranging from 400 to 900 nmol/min/mg of protein depending on preparations. Fig. 5 shows the representative data.

To confirm that ATPase activity is derived from purified ABCA1, a baculovirus for ABCA1 K939M-K1952M mutant, in which lysine 939 and lysine 1952 in the Walker A motif of nucleotide binding folds were replaced by methionines, was constructed. These lysines were reported to be indispensable for ATP hydrolysis of ABC proteins (29). Purified ABCA1 K939M-K1952M protein showed little ATPase activity, less than 10 nmol/min/mg of protein (Fig. 5). These results suggest that the purified ABCA1 reconstituted into soybean lipids shows ATPase activity.

Lipid Specificity in Stimulating ABCA1 ATPase—It has been reported that purified MDR1 shows ATPase activity only after reconstitution in liposomes and stimulation by transport substrates (30). We compared the ATPase activity of ABCA1 and MDR1 purified with the same procedure (Fig. 6*A*). Purified human MDR1 (supplemental Fig. 3) reconstituted in soybean lipids showed minimum ATPase activity, which was strongly stimulated only after the addition of verapamil, a transport substrate for MDR1. In contrast, ABCA1 showed significant ATPase activity even before reconstitution, and the ATPase activity was stimulated by the addition of soybean lipids. Verapamil did not affect the ATPase activity of ABCA1 either before or after reconstitution (data not shown).

We hypothesized that lipids themselves stimulate ABCA1 ATPase activity. If this is the case, we may speculate that there is lipid specificity

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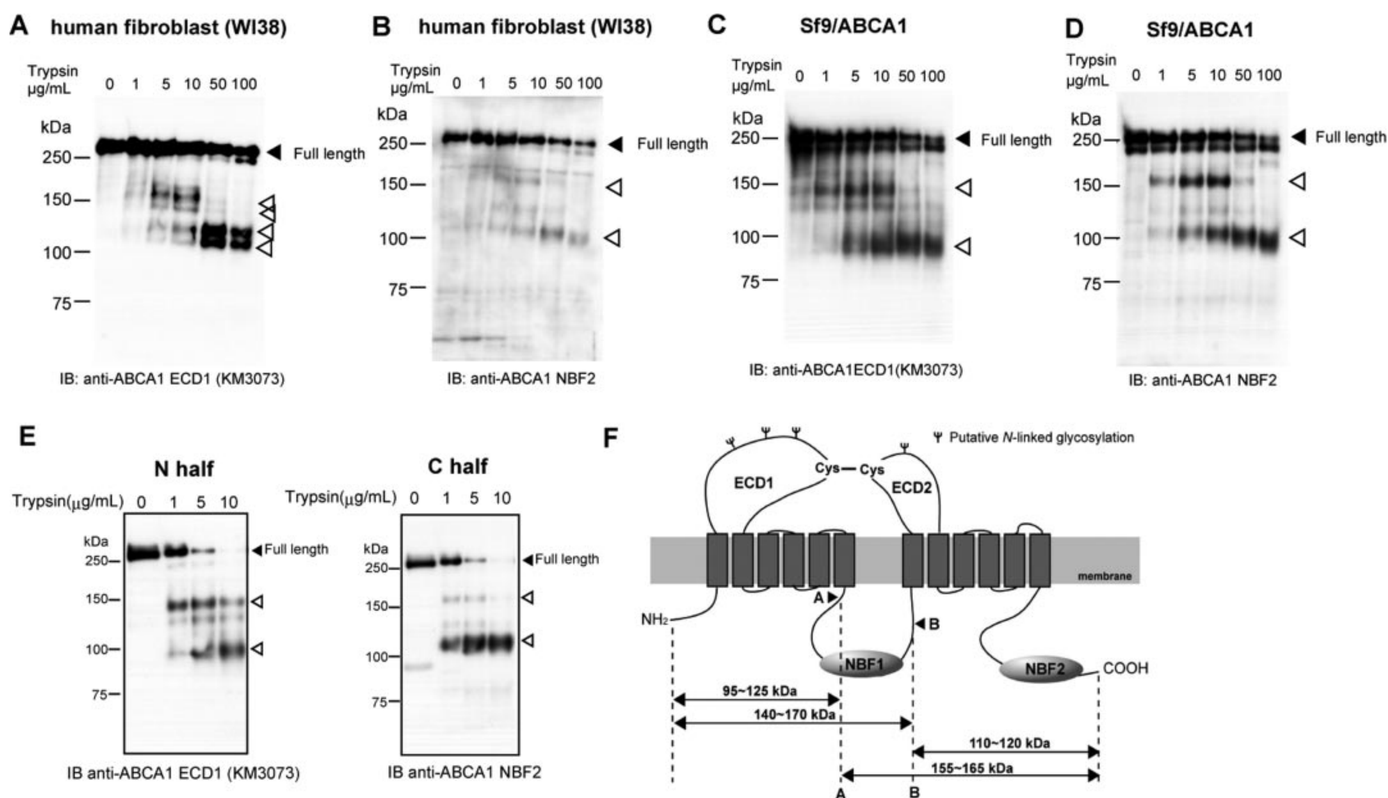

FIGURE 3. **Trypsin-limited digestion of ABCA1.** *A* and *B*, human fibroblast (WI-38) membranes treated with the represented concentration of trypsin were separated with 7% polyacrylamide gel under reducing conditions. ABCA1 was detected by anti-ABCA1 ECD1 monoclonal antibody (KM3073) (*A*) or anti-ABCA1 NBF2 polyclonal antibody (*B*). Full-length ABCA1 is indicated by a *black triangle* and limited digested ABCA1 fragments by *white triangles*. *C* and *D*, Sf9 membranes expressing ABCA1 were treated and detected as described above. *E*, purified ABCA1 was treated with 1, 5, and 10 μg/ml trypsin and detected as described above. *F*, schematic diagram of typsin-limited digestion of ABCA1 and the molecular size of produced fragments.

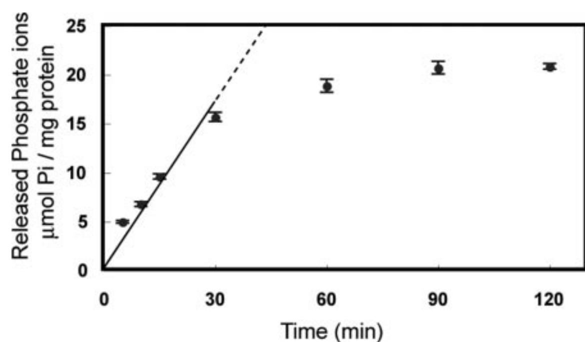

FIGURE 4. **Dependence of ATPase activity on the reaction time.** Purified ABCA1 was reconstituted with soybean lipids as described under "Experimental Procedures." Released inorganic phosphate ions were quantified. Each data point is the mean ± S.D. ($n = 4$).

in stimulating ABCA1 ATPase activity. To examine this possibility, we prepared liposomes composed of synthetic phospholipids with various head groups or acyl chains. ABCA1 was first reconstituted in liposomes composed of 16:0–18:1 PC and 16:0–18:1 PS in various ratios (Fig. 6*B*). ATPase activity was lowest when reconstituted in 100% PS liposomes, increased with the increasing ratio of PC, and was highest when liposomes contained more than 70% PC. In consequence, ABCA1 showed almost 2-fold higher ATPase activity in PC liposomes than in PS liposomes. Next, ABCA1 was reconstituted in liposomes composed of 16:0–18:1 PC and other phospholipid species such as SM, PE, or PG in various ratios (Fig. 6*C*). The content of SM in PC liposomes did not significantly affect the ATPase activity of ABCA1; however, PG inhibited the ATPase activity of ABCA1 depending on its content, and PE inhibited ATPase activity by 50%, even at 20% content (Fig. 6*C*). These results suggest that the ATPase activity of ABCA1 is stimulated preferentially by phospholipids with choline head groups, PC and SM. We also explored the preference of acyl chain length and the number of double bonds of phospholipids (Fig. 6*D*). When ABCA1 was reconstituted in liposomes containing PC (16:0–18:1), it showed higher ATPase activity than when reconstituted in liposomes containing PC (18:0–18:0), PC (20:0–20:0), or PC (18:0–18:1). These results suggest that there is some acyl chain preference in stimulating ABCA1 ATPase activity.

Effect of Sterols on ABCA1 ATPase Activity—As ABCA1 is involved in loading cellular free cholesterol onto apoA-I, we examined the effect of cholesterol and other sterols on ABCA1 ATPase activity. Purified ABCA1 was reconstituted in 16:0–18:1 PC and 16:0–18:1 PS (8:2) liposomes containing various amounts of cholesterol, and ABCA1 ATPase was analyzed. ABCA1 ATPase activity decreased in a dose-dependent manner of cholesterol and was reduced by 25% in the presence of 20% cholesterol (Fig. 7*A*). The structure specificity of sterols in inhibiting ABCA1 ATPase was examined. β-Sitosterol and campesterol showed a similar inhibitory effect with cholesterol (Fig. 7*B*). Brassicasterol and ergosterol showed intermediate effects, but stigmasterol scarcely inhibited ABCA1 ATPase.

Beryllium Fluoride and Glibenclamide Inhibit ABCA1 ATPase Activity—ABCA1 ATPase activity was completely abolished in the absence of magnesium ions (Fig. 8). It was also inhibited efficiently by beryllium fluoride, a phosphate analogue, but not by vanadate, another phosphate analog. Glibenclamide, which has been used as an inhibitor of ABCA1 in apoA-I-dependent cellular cholesterol efflux (3, 31, 32), suppressed the ATPase activity of ABCA1 in a dose-dependent manner,

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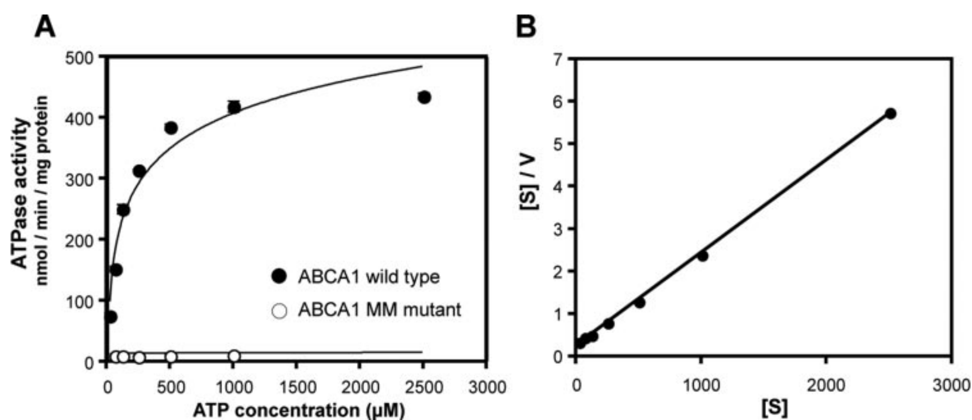

FIGURE 5. **ATP dependence of ATPase activity of wild-type and mutant ABCA1.** *A*, ATPase activity was measured in the presence of various concentrations of MgATP. ●, wild type; ○, K939M-K1952M mutant. The experiment was performed in triplicate, and data are represented as the mean ± S.D. *B*, data were plotted to Michaelis-Menten formula. The modified Lineweaver-Burk plot, *[S]* − *[S]/V* plot was used to determine kinetic parameters. The K_m for ATP and the V_{max} of ATP hydrolysis are 0.112 mM and 455 nmol/min/mg of protein, respectively.

FIGURE 6. **Lipid dependence of ATPase activity of ABCA1.** *A*, the ATPase activity of purified ABCA1 and MDR1 in the absence or presence of soybean lipids and verapamil. The experiment was performed in triplicate and data are represented as the mean ± S.D. ***B*****,** the ATPase activity of ABCA1 reconstituted in POPC:POPS liposomes. Purified ABCA1 was reconstituted in various ratios of POPC:POPS liposomes, and ABCA1 ATPase activity was measured as described under "Experimental procedures." *C*, the ATPase activity of ABCA1 reconstituted in POPC:DPPE liposomes, POPC:DPPG liposomes, and POPC:SM liposomes containing various amounts of PE, PG, or SM. Relative ATPase activity to the activity in POPC liposomes is presented. *D*, the ATPase activity of ABCA1 reconstituted in liposomes containing 80% PC with various acyl chains and 20% PS (16:0, 18:2). Relative ATPase activity to the activity in POPC liposomes is presented. Experiments were performed three times, and representative data are shown.

and the stimulatory effect of phospholipids on ABCA1 ATPase was reduced by 35% at 0.8 mM (Fig. 9).

Effect of ApoA-I on ABCA1 ATPase Activity—Cellular cholesterol and PL efflux mediated by ABCA1 is fully dependent on lipid-free apoA-I. To examine the effect of apoA-I on ABCA1 ATPase activity, apoA-I was added to the suspension either before or after liposome formation. However, we found no clear effects of apoA-I on the ATP hydrolysis of purified ABCA1 (Fig. 8). We also examined the effect of apoA-I by using synthetic PC and in the presence of cholesterol, but we found no clear effects either (data not shown).

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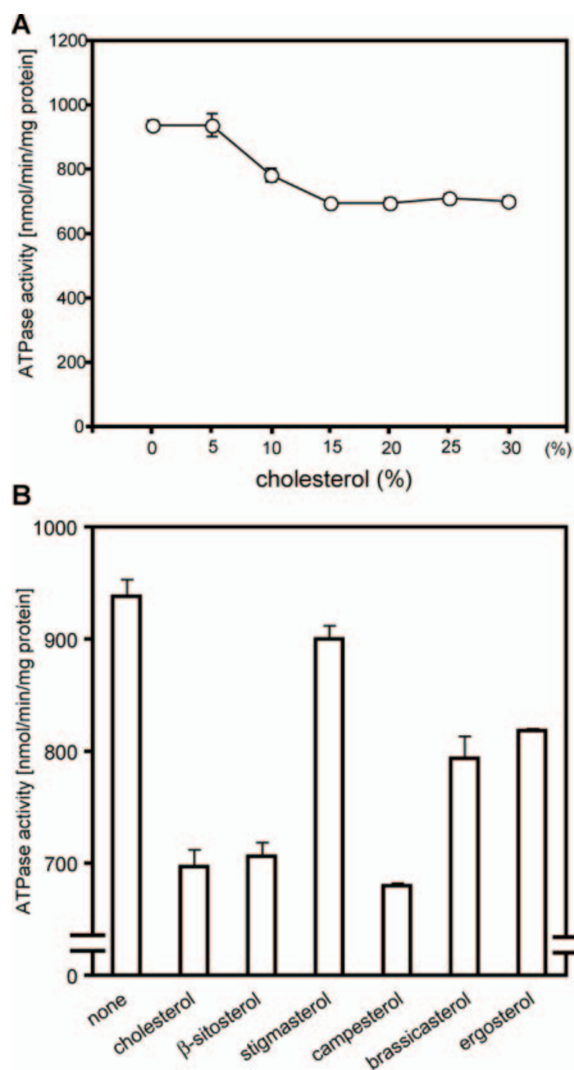

FIGURE 7. **Mild inhibition of ABCA1 ATPase activity by cholesterol.** *A*, ATPase activity of ABCA1 reconstituted in POPC:POPS (80:20) liposomes containing various concentrations of cholesterol (w/w %). *B*, effects of sterols (20%) on ABCA1 ATPase activity. Experiments were performed twice in triplicate. Data are presented as the mean ± S.D.

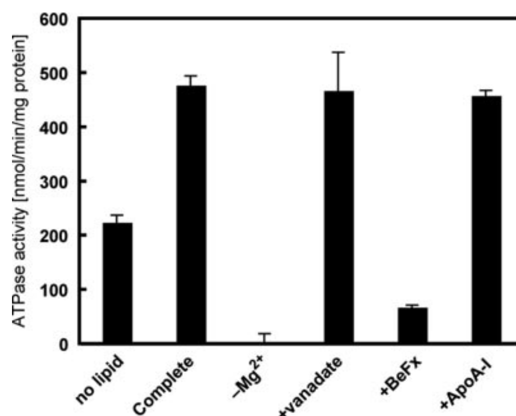

FIGURE 8. **Effects of phosphate analogs, vanadate, beryllium fluoride (*BeFx*), and apoA-I on ABCA1 ATPase activity.** The ATPase activity of ABCA1 reconstituted in soybean lipids was analyzed in the presence of 1 mM vanadate, 0.25 mM beryllium fluoride, and 500 ng of purified apoA-I from human plasma or in the absence of magnesium ions. Data are presented as the mean ± S.D. Experiments were performed three times, and representative data are shown.

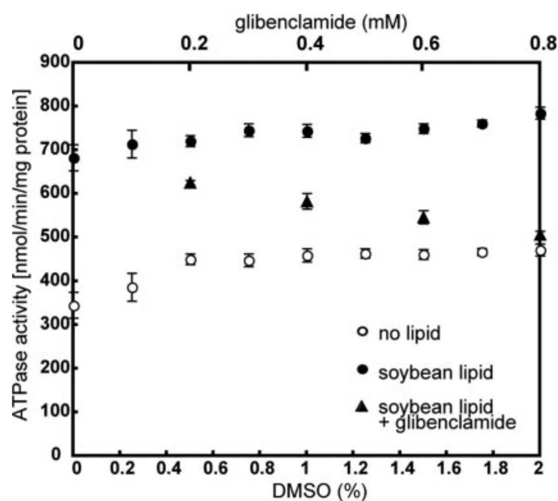

FIGURE 9. **Effects of glibenclamide on ABCA1 ATPase activity.** The ATPase activity of ABCA1 reconstituted in soybean lipid in the presence or absence of glibenclamide (0.2–0.8 mM) was analyzed. The concentrations of dimethyl sulfoxide (*DMSO*), used as a solvent, in the reaction mixture were also indicated. Data are presented as the mean ± S.D.

DISCUSSION

Some of the most critical questions for ABCA1 function in nascent HDL formation are whether ABCA1 is a phospholipid transporter or not, which PL is the substrate for ABCA1, and whether cholesterol is a direct substrate for ABCA1. To answer these questions, we expressed human ABCA1 in insect cells, purified it, and analyzed ATP hydrolysis by this purified ABCA1 in this study.

First we confirmed that ABCA1 expressed in the Sf9 membrane retained the correct conformation. Trypsin limited digestion of purified ABCA1, producing N-terminal 140- and 95-kDa fragments and C-terminal 155- and 110-kDa fragments. These fragments were corresponded well with fragments produced from ABCA1 endogenously expressed in human fibroblast WI-38 cells by cleaving at just after the sixth transmembrane α-helix and just before the seventh transmembrane α-helix. These results suggested that purified ABCA1 retained conformation similar to endogenously expressed ABCA1.

Detergent-soluble ABCA1 showed significant basal ATPase activity even before reconstitution in liposomes. This feature was different from the multidrug transporter MDR1, which showed very low activity without reconstitution in liposomes. As ABCA1 is thought to be involved in PC transport, as discussed later, the binding affinity of PC to ABCA1 might be higher than to MDR1, and purified ABCA1 might contain endogenous PLs of Sf9 that were not removed during the purification. This could possibly account for the relatively high base-line level of ATPase activity. Intramolecular disulfide formation could also contribute to the stability of the structure and high basal ATPase activity. Indeed, ABCA1 was very stable in the refrigerator after purification and showed more than 70% activity even after a week (data not shown).

Purified ABCA1 showed robust ATPase activity when reconstituted in liposomes made of synthetic PC. ABCA1 showed lower ATPase activity when reconstituted in liposomes containing PS, PE, or PG compared with in PC liposomes. ABCA1 also showed high ATPase activity in SM liposomes. Among PC species, PC containing 16:0 and 18:1 acyl chains was best for supporting ABCA1 ATPase. These results suggest that the ATPase activity of ABCA1 is stimulated preferentially by phospholipids with choline head groups, PC and SM. It has been reported that the ATPase activity of ABCA4 (ABCR), which has 64.5% homology in amino acid sequences with ABCA1, is stimulated by all-*trans*-retinal especially in the presence of PE (33). The reported ATPase activity of ABCA4 (400–700 nmol/min/mg of protein) (33) was comparable with

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that of ABCA1 (400–900 nmol/min/mg of protein). Furthermore, the ATPase activity of ABCA4 was stimulated by *N*-retinylidene-PE but not by *N*-retinyl-PE (33). These results suggest that the head group of PE is recognized by ABCA4. The physiological function of ABCA4 is thought to remove *N*-retinylidene-PE from disc membranes in an ATP hydrolysis-dependent manner after photobleaching of rhodopsin (34, 35). These results support the idea that choline phospholipids are the primary substrates for ABCA1, and translocating them in the plasma membrane is the physiological function of ABCA1. Szakacs *et al.* (12) speculated that ABCA1 is not an effective active transporter because vanadate-sensitive ATP hydrolysis was not detected in Sf9 membranes expressing ABCA1. As the ATPase activity of ABCA1 is not inhibited by vanadate as discussed later, purified protein should be used to reveal the function of ABCA1.

The ATPase activity of purified ABCA1, reconstituted in 16:0–18:1 PC and 16:0–18:1 PS (8:2) liposomes, was reduced by the addition of cholesterol and decreased by 25% in the presence of 20% cholesterol (Fig. 7*A*). β-Sitosterol and campesterol, which do not have a double bond in the acyl chain as cholesterol (supplemental Fig. 4), showed a similar inhibitory effect with cholesterol, but stigmasterol scarcely affected ABCA1 ATPase. These results suggest several possibilities, such as (i) cholesterol directly interacts with ABCA1 and becomes a burden on the conformational changes of ABCA1, (ii) cholesterol interacts with PLs, which affects the interaction between PLs and ABCA1, and (iii) sterols affect membrane fluidity, which suppresses the ATPase activity of ABCA1. If the first explanation is true, these results might support a concurrent process model in which both FC and PL are directly transported by ABCA1; however, it is clear that further studies are necessary to reveal the interaction between cholesterol and ABCA1.

Glibenclamide, a sulfonylurea derivative, has been reported to be an effective inhibitor of apoA-I-dependent cellular cholesterol efflux (31, 32) and the interaction between apoA-I and ABCA1 (3). We examined the effect of glibenclamide on ABCA1 ATPase and found that glibenclamide suppressed the ATPase activity of ABCA1 in a dose-dependent manner (Fig. 9). These results suggest that glibenclamide inhibits apoA-I-dependent cellular cholesterol efflux by suppressing ABCA1 ATPase activity.

Vanadate, a phosphate analog, did not inhibit ABCA1 ATPase, although the ATPase activity of many ABC proteins, such as MDR1, is efficiently inhibited by vanadate (36). This is well consistent with the report by Szakacs *et al.* (12) that vanadate-sensitive ATP hydrolysis was not detected in Sf9 membranes expressing ABCA1. It was also reported that vanadate did not affect apoA-I-dependent PL and cholesterol efflux from ABCA1-expressing cells (3). Beryllium fluoride, another phosphate analog, efficiently inhibited ATP hydrolysis by ABCA1. Both vanadate and beryllium fluoride efficiently inhibited the ATPase activity of multidrug transporters, MDR1 (ABCB1), MRP1 (ABCC1), and MRP2 (ABCC2) via forming stable inhibitory intermediates during the ATP hydrolysis cycle (36–39); however, ATP hydrolysis by transporter associated with antigen processing (TAP) (40), a peptide antigen transporter, and ABCC6 (41), whose mutations cause pseudoxanthoma elasticum, is inhibited by beryllium fluoride but not by vanadate. The NBFs of each ABC protein may differ slightly in structure after ATP hydrolysis, although the amino acid sequences of NBFs of ABC proteins are well conserved.

Another critical issue in ABCA1-mediated pre-βHDL formation is the role of apoA-I. Cellular cholesterol and PL efflux mediated by ABCA1 is fully dependent on lipid-free apoA-I. As the ABCA1 K939M mutant, whose Walker A lysine residue is substituted by methionine, is reported to be defect in its interaction with apoA-I, we expected that apoA-I would affect the ATP hydrolysis of ABCA1; however, we found no clear effects of apoA-I on the ATP hydrolysis of purified ABCA1 either before or after reconstitution in liposomes under our examination conditions (Fig. 8). ABCA1 reconstituted in liposomes was efficiently cleaved by trypsin at sites A and B (Fig. 3*F*; data not shown) and showed robust ATPase activity, and proteoliposomes prepared under the conditions were considered to be mainly inside-out vesicles. Therefore, apoA-I was added during liposome preparation to be contained in liposomes. ApoA-I was also added after reconstitution and in the presence of synthetic PC and cholesterol, but we found no clear effects on the ATPase activity either (data not shown). It was reported that ABCA1 exerted translocase activity without adding apoA-I as assessed by annexin V binding assay and by using *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-labeled PL (5, 42, 43). ABCA1 might be able to translocate lipids without interacting with apoA-I.

In summary, purified ABCA1 showed robust ATPase activity when reconstituted in liposomes made of synthetic PC. As ABCA1 showed lower ATPase activity when reconstituted in liposomes containing PS, PE, or PG, and some specificity in acyl chain species, choline phospholipids are suggested to be the primary substrates for ABCA1. Cholesterol, as well as β-sitosterol and campesterol, mildly suppressed ABCA1 ATPase. If these results suggest that cholesterol is a burden on the conformational changes of ABCA1, it would support a concurrent process model in which both FC and PL are directly transported by ABCA1. Glibenclamide suppressed ABCA1 ATPase, suggesting that it inhibits apoA-I-dependent cellular cholesterol efflux by suppressing ABCA1 ATPase activity. This study with purified human ABCA1 provides the first biochemical basis of the mechanism for HDL formation mediated by ABCA1, although further study is necessary to understand entirely the roles of ABCA1 and apoA-I in HDL formation.

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Purification and ATPase Activity of Human ABCA1

Kei Takahashi, Yasuhisa Kimura, Noriyuki Kioka, Michinori Matsuo and Kazumitsu Ueda

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