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Purification and ATPase Activity of Human ABCA1*[S]

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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.

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[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.

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

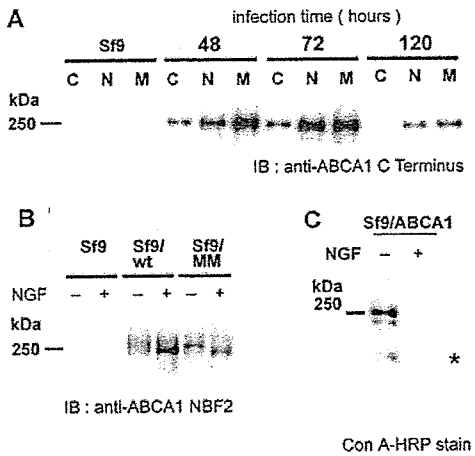

FIGURE 1. **Membrane localization and glycosylation of human ABCA1 expressed in Sf9 cells.** *A*, Western blotting of human ABCA1-TATH protein expressed in Sf9 cells. Proteins in cytosolic (*C*), nuclear (*N*), and microsomal (*M*) fractions (10 μg) were separated on 7% SDS-PAGE gel. ABCA1-TATH was detected 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

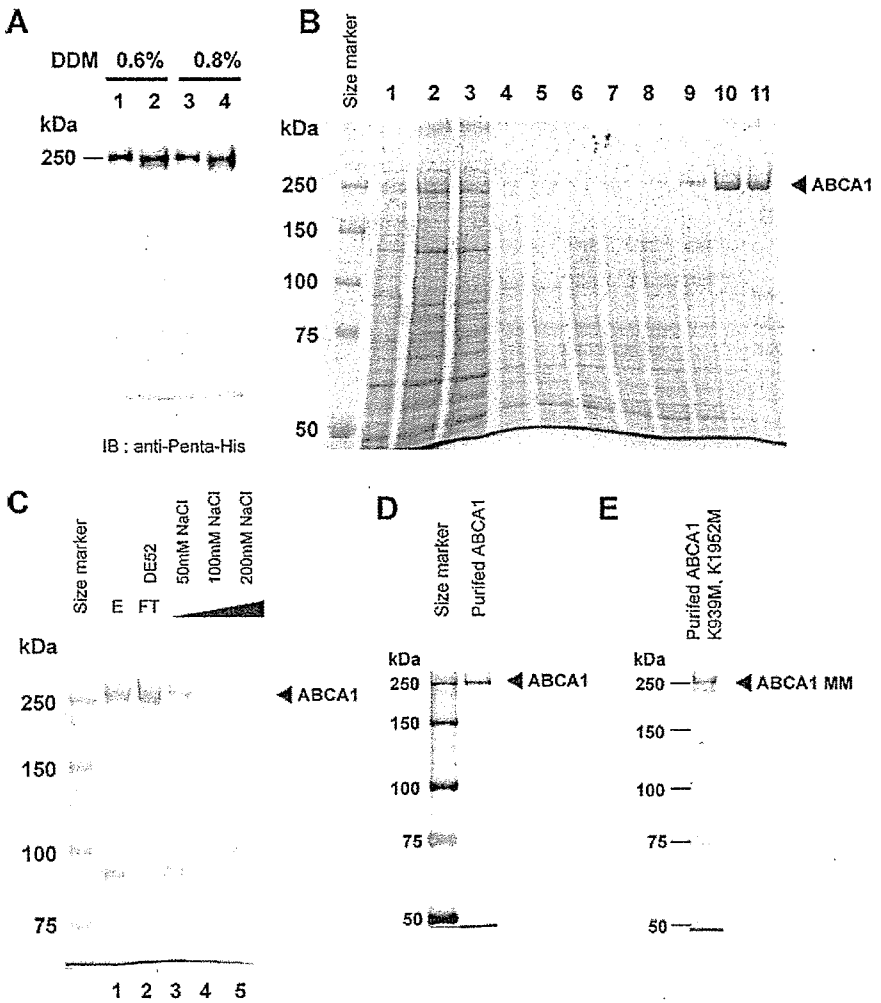

FIGURE 2. **Purification of ABCA1.** *A*, Sf9 membranes were extracted with 0.6% (*lanes 1* and *2*) and 0.8% DDM (*lanes 3* and *4*). *Lanes 1* and *3*, soluble fraction; *lanes 2* and *4*, insoluble fraction. Proteins (10 μg) were separated on 7% polyacrylamide gel, and ABCA1-TATH was detected with 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

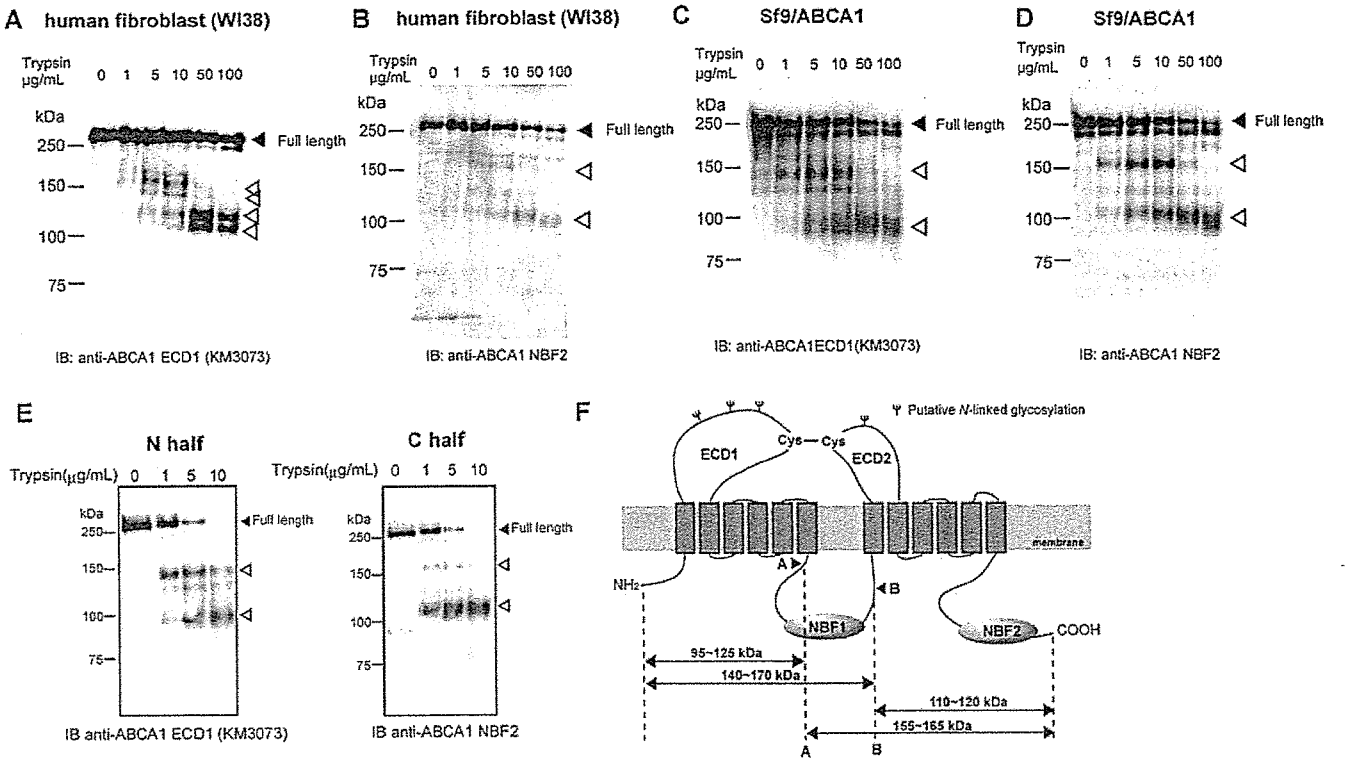

FIGURE 3. **Trypsin-limited digestion of ABCA1.** *A* and *B*, human fibroblast (WI-38) membranes treated with the represented concentration of trypsin were separated with 7% polyacrylamide gel under reducing conditions. ABCA1 was detected by anti-ABCA1 ECD1 monoclonal antibody (KM3073) (*A*) or anti-ABCA1 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.

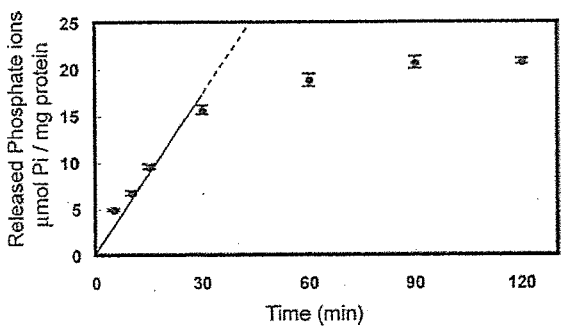

FIGURE 4. **Dependence of ATPase activity on the reaction time.** Purified ABCA1 was reconstituted with soybean lipids as described under "Experimental Procedures." Released inorganic phosphate ions were quantified. Each data point is the mean ± S.D. ($n = 4$).

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,

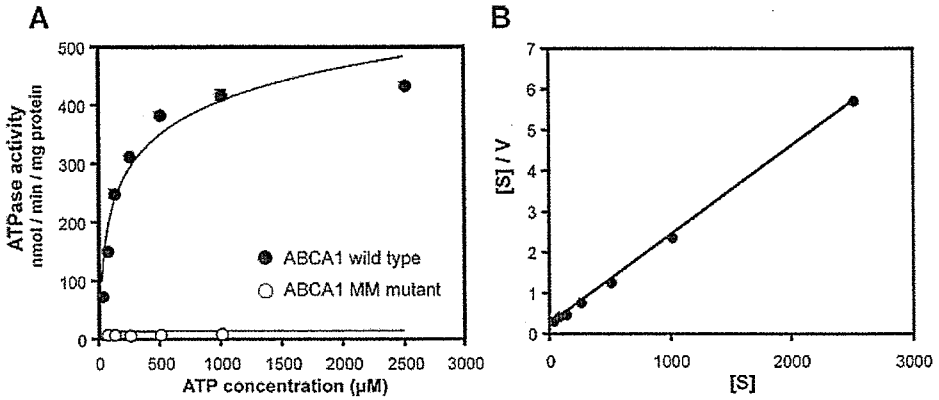

FIGURE 5. **ATP dependence of ATPase activity of wild-type and mutant ABCA1.** *A*, ATPase activity was measured in the presence of various concentrations of MgATP. ●, wild type; ○, K939M-K1952M mutant. The experiment was performed in triplicate, and data are represented as the mean ± S.D. *B*, data 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.

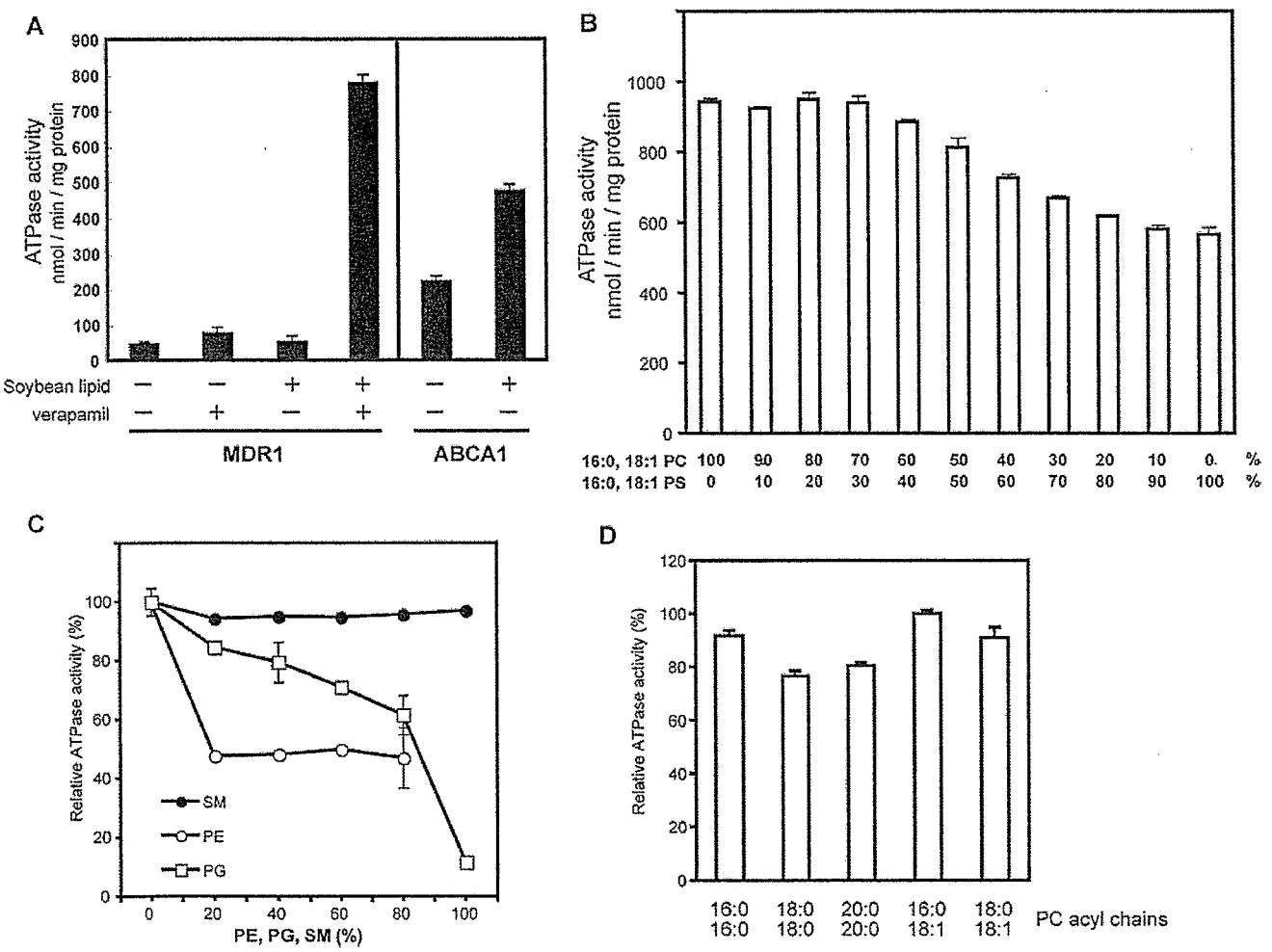

FIGURE 6. **Lipid dependence of ATPase activity of ABCA1.** *A*, the ATPase activity of purified ABCA1 and MDR1 in the absence or presence of soybean lipids and verapamil. The experiment was performed in triplicate and data are represented as the mean ± S.D. *B*, the ATPase activity of ABCA1 reconstituted 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).

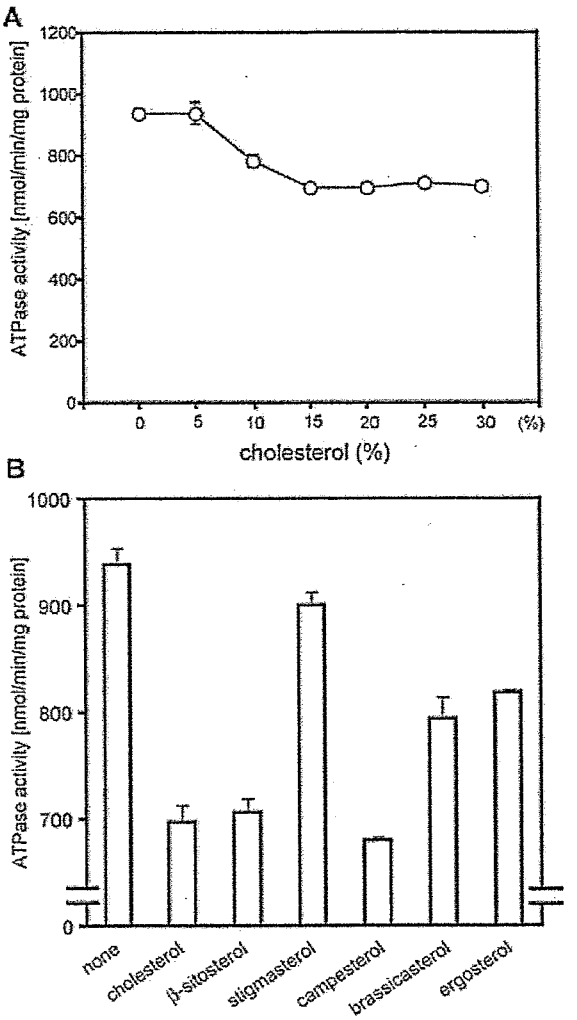

FIGURE 7. **Mild inhibition of ABCA1 ATPase activity by cholesterol.** *A*, ATPase activity of ABCA1 reconstituted in POPC:POPS (80:20) liposomes containing various concentrations of cholesterol (w/w %). *B*, effects of sterols (20%) on ABCA1 ATPase activity. Experiments were performed twice in triplicate.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

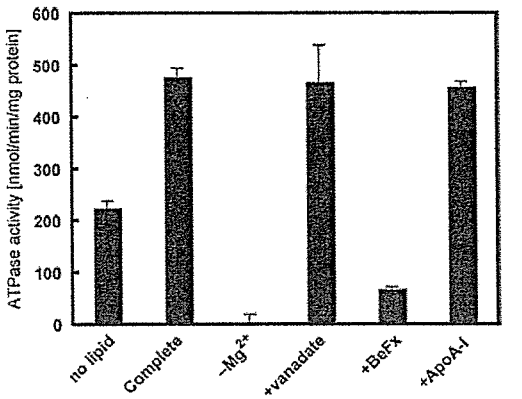

FIGURE 8. **Effects of phosphate analogs, vanadate, beryllium fluoride (*BeFx*), and apoA-I on ABCA1 ATPase activity.** The ATPase activity of ABCA1 reconstituted in soybean lipids was analyzed in the presence of 1 mM vanadate, 0.25 mM beryllium fluoride, and 500 ng of purified apoA-I from human plasma 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.

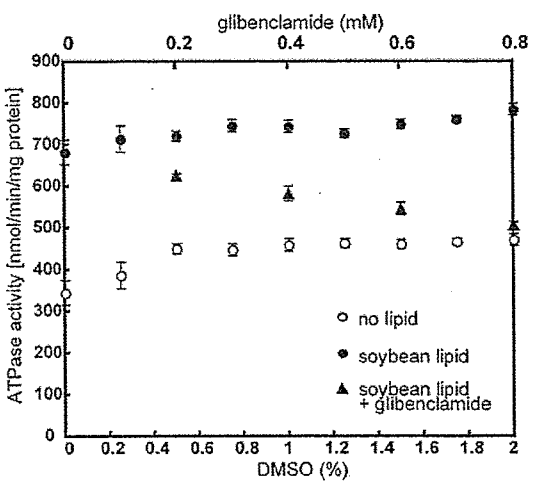

FIGURE 9. **Effects of glibenclamide on ABCA1 ATPase activity.** The ATPase activity of ABCA1 reconstituted in soybean lipid in the presence or absence of glibenclamide (0.2–0.8 mM) was analyzed. The concentrations of dimethyl sulfoxide (*DMSO*), used as a solvent, in the reaction mixture were also indicated.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

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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Heterologous expression of a mammalian ABC transporter in plant and its application to phytoremediation

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Key words: ABC transporter, cadmium, complex pollutants, daunorubicin, MRP1, phytoremediation

Abstract

Mammalian ATP-binding cassette (ABC) transporters involved in the multidrug-resistance of cancer cells can efflux cytotoxic compounds that show a wide variety of chemical structures and biological activities. Human multidrug resistance-associated protein (hMRP1) is one of the most intensively studied ABC transporters and many substrates have been identified, including both organic and inorganic compounds. In an attempt at novel 'transport engineering' using hMRP1 as a molecular pump, we established transgenic tobacco plants that showed clear resistance to cadmium and daunorubicin, although they were not resistant to etoposide, another known substrate of hMRP1. When expressed in tobacco cells, hMRP1 protein was localized at vacuolar membrane, while members of the MRP family are localized at plasma membrane in mammalian cells to reduce the cellular accumulation of various drugs. Thus, the hMRP1-expressing tobacco cells were able to take up these substrates across the tonoplast and sequestrate them in the vacuolar matrix. These results suggest that it may be possible to use the transgenic tobacco in phytoremediation, where a single transformation with an ABC transporter with broad substrate specificity should be effective for extracting various environmental pollutants including both organic and inorganic compounds, and accumulate them in the plant body. This should be advantageous for the remediation of a complex polluted environment, which is commonly found in the real world.

Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MS, Murashige and Skoog; MRP, multidrug resistance-associated protein; PMSF, phenylmethylsulfonyl fluoride; PPase, pyrophosphatase; PVDF, polyvinylidene difluoride; PVPP, polyvinylpolypyrrolidone; YCF1, yeast cadmium factor 1

Introduction

Recent developments in molecular biology enable us to alter the biological functions of plants, e.g. engineering of primary and secondary metabolism, and this has become a popular technique in plant research. There are many reports on the increase of valuable natural products through the overexpression of biosynthetic genes with a strong promoter and a suitable signal sequence to control the preferred subcellular localization (Sato *et al.*, 2001; Verpoorte and Memelink, 2002; Ohara *et al.*, 2004; Yazaki, 2004). On the other hand, antisense and RNAi technologies are often used for gene silencing, by which the expression of a biosynthetic step can be blocked to reduce the production of an undesired metabolite (Mol *et al.*, 1990; Vaucheret *et al.*, 2001; Wang *et al.*, 2001). In contrast to these successful examples of metabolic engineering, there have been few trials to alter the transport ability by expressing a transporter molecule, i.e. 'transport engineering', to achieve the high accumulation of endogenous metabolites or to increase resistance to environmental stresses, such as heavy metals (Hirschi *et al.*, 2000; Pence *et al.*, 2000; Song *et al.*, 2003).

Among many transporter proteins, ATP-binding cassette (ABC) transporter is a large protein family with a wide distribution from bacteria to human, in which they are involved in the uptake or efflux of various compounds across biomembranes (Higgins, 1992). The properties of ABC transporters have been intensively studied in mammalian systems, particularly in cancer research, since the multidrug-resistance of cancer cells is often mediated by such ABC transporters functioning as drug efflux pumps that show very broad substrate specificity (Deeley and Cole, 1997; Ambudkar, *et al.*, 1999).

In ABC transporter studies, while several heterologous host organisms, e.g. insect cells and yeast, have been used to express these genes (Tommasini *et al.*, 1996; Shitan *et al.*, 2003; Terasaka *et al.*, 2005), plants have rarely been reported as a host organism. Plant cells have some advantages as a host organism for the expression of foreign genes, especially for eukaryotic membrane protein, since plants have developed membrane systems, many established vectors are available, transformation methods are well-established for model plants such as *Arabidopsis*, and appropriate knock-out lines are now available from many institutes.

In this study, we attempted to confer resistance to environmental stress to plants through the expression of a mammalian ABC transporter, multidrug resistance-associated protein (hMRP1) (Zaman *et al.*, 1994; Loe *et al.*, 1996), which was involved in the multidrug-resistance of cancer cells (Muller *et al.*, 1994; Jedlitschky *et al.*, 1996). We chose hMRP1 as a model transporter because it is one of the most intensively studied ABC transporters and therefore abundant biochemical data resources are available, including regarding its substrate specificity. Tobacco was used as a host plant in the 'transport engineering' experiment of this study, since it has a large plant mass, which is advantageous for clarifying the subcellular localization of this heterologous ABC transporter and also for clarifying the ability of transgenic plants to accumulate or efflux its substrates. In this report, we describe the behavior of mammalian ABC transporter in plant cells and the potential for using transgenic plants for phytoremediation.

Materials and methods

Plant materials and reagents

Tobacco, *Nicotiana tabacum* (cv. Samsun NN), was used as the host plant to express *hMRP1*. Control and transgenic tobacco plants were grown under controlled conditions as described elsewhere (Takeda *et al.*, 1990). They were aseptically maintained in 15 cm-high plastic pots. Callus and cell suspension cultures of tobacco were subcultured in modified Murashige and Skoog's (MS) medium containing 10^{-5} M naphthaleneacetic acid and 10^{-6} M kinetin (Takeda *et al.*, 1990).

Daunorubicin and etoposide were purchased from Wako Pure Chemicals (Japan). Radiochemicals were purchased from Amersham Biotech. Other common chemical reagents were purchased from Wako Pure Chemicals (Japan), Nacalai (Japan), and Sigma.

Transformation of tobacco

Leaf disks of axenic plantlets of *N. tabacum* (cv. Samsun NN) were used as a plant material for transformation. The plantlets were maintained on

agar media containing a half-concentration of MS medium inorganic components, 1.5% sucrose, and 0.8% agar at 25 °C under continuous light (100 mmol quanta m^{-2} s^{-1}). The mature leaves were used for standard *Agrobacterium*-mediated transformation (Horsh *et al.*, 1985).

Construction of expression vector

The plasmid pJ3Ω-MRP containing full-length cDNA of the human *mrp1* gene (*hmrp1*) (Zaman *et al.*, 1994) was modified for subcloning into the binary vector pBin-El2-GUS, which has a CaMV 35S promoter equipped with a tandem enhancer sequence El2 and a hygromycin-resistance gene as a selection marker (Yazaki *et al.*, 2001). The *Sal* I site at the 5′-end and the *Not* I site at the 3′-end of the *mrp1* cDNA insert were changed to *Xba* I and *Sac* I sites, respectively, via restriction digest and ligation of the corresponding linkers. The full-length *hmrp1* cDNA excised by these restriction sites was then subcloned into the binary vector by replacement with the GUS gene, to complete the expression vector pBI-El2-MRP1. *Agrobacterium tumefaciens* (LBA4404) was transformed with this expression vector by electroporation.

Northern analysis of hMRP1 *expression*

Transformed tobacco plants were grown at 25 °C under continuous light (80 $\mu E/m^2$ s) with fluorescent lamps as wild type tobacco, from which green leaves were cut to extract total RNA. Total RNA was extracted from frozen leaves (0.3 g) by the aurintricarboxylic acid (ATA) method (Skidmore and Beebee, 1989). Northern blot hybridization was carried out according to the standard protocol using 10 μg of total RNA samples. The *Bam* HI/*Eco* RI fragment of *hmrp1* (3.0 kb) was used as a probe, with which no detectable background was observed in the RNA blot of wild type tobacco. The 18S rDNA of broad bean was used as a load control (Yakura and Tanifuji, 1983).

Western analysis of hMRP1 *in tobacco*

We used a western blot protocol that was often used to detect ABC transporters in mammalian cells (Bradley *et al.*, 1989). The protein extraction procedure was performed at 4 °C. Tobacco leaf (0.5 g) was homogenized with 1.5 ml homogenization buffer composed of 50 mM HEPES-KOH (pH 7.5), 0.1% BSA, 1 mM PMSF, 2 mM DTT, 1% (w/v) PVPP, 0.1 mg/ml butylated hydroxytoluene, and 0.25 M sucrose in mortar. After filtration with Miracloth (Merck), the homogenate was centrifuged at 7000 *g* for 15 min to yield the supernatant, which was then ultracentrifuged at 100 000*g* for 30 min (TL-100, Beckman). The supernatant was used for SDS-PAGE whereas the pellet was resuspended with resuspension buffer that consisted of 5 mM Tris–HCl (pH 7.2), 2 mM EDTA, 250 mM sucrose, and 1% (w/v) Triton X-100. Protein content was measured by the method of Bradford, and 30 μg of sample was mixed with 1/4 volume of 5× Ling's solubilizing buffer. The mixture was allowed to stand at 50 °C for 10 min and the same volume of 2× Ling's urea buffer was added for loading. SDS-PAGE was performed as described elsewhere (Bradley *et al.*, 1989). The amount of protein loaded on the gel and the separation pattern was monitored by staining with Coomassie Brilliant Blue (CBB) according to the standard procedure. The transfer to PVDF membrane (Millipore) was performed with a Semi-dry transfer cell (Bio-Rad), and the membrane was treated with the primary antibody MRPr1 (Nichirei, at 3000 dilution). Anti-rat IgG (H + L) peroxidase conjugate (Calbio, at 3500 dilution) and Renaissance western blot chemiluminescence reagent plus (NEN Life Science) were used as the secondary antibody and for the visualization of signal, respectively.

Sucrose density gradient of tobacco microsomal fraction

Fresh leaves (50 g) of transgenic plant clone (P-3) of ca. 30 cm in height were homogenized in 30 ml of 0.1 M Tris–HCl buffer (pH 8.0) containing 3.3 mM DTT, 5 mM EDTA, 0.5% (w/v) PVPP, 10% glycerol, 0.13% BSA, 150 mM KCl, and protease inhibitor mixture. Protease inhibitor mixture was composed of PMSF (1 mM), leupeptin (10 μg/ml), aprothinin (2 μg/ml), and pepstatin (2 μg/ml) at the final concentration. After filtration of the homogenate through Miracloth (Merck), the supernatant obtained by centrifugation at 8000 *g* was further ultracentrifuged at 100 000*g* for 40 min at 4 °C. The pellet was resuspended in 10 mM Tris–HCl buffer (pH 7.6) containing 1 mM DTT, 1 mM EDTA, 10% glycerol and protease inhibitor mixture, and

fractionated through a non-linear density gradient of 20–60% sucrose by centrifugation at 100 000*g* for 2 h. The fractionated membrane was sampled by pipetting, and recovered by ultracentrifugation for 40 min. Western blot analysis was carried out as described above. The polyclonal antibodies against tobacco plasma membrane H^+-ATPase (W1D) (Morsomme *et al.*, 1998) and *Arabidopsis* vacuolar H^+-PPase (AVP1) (Mitsuda *et al.*, 2001) were generous gifts from Dr M. Boutry of the Université Catholique de Louvain and Dr M. H. Sato of the Faculty of Integrated Human Sciences of Kyoto University, respectively.

Analyses of tolerance to drugs

Suspension-cultured cells of transgenic tobacco were used to examine the tolerance to cadmium and drugs which were known to be substrates of hMRP1, i.e. daunorubicin (final concentration 0–120 μM), etoposide (0–90 μM) and cadmium chloride (0–100 μM). To avoid the influence of somaclonal variation, we used cultured cells of the same culture passage after callus induction for both transgenic and wild type tobacco. By subculturing every 2 weeks, the cell cultures were stabilized and 3–5 passages after the initiation of suspension cultures were used in these experiments. Each drug was added after filter-sterilization to 30 ml of fresh modified MS medium (in 100 ml conical flask) (Takeda *et al.*, 1990), to which 1 g of fresh tobacco cells were inoculated. Cells were cultured on a rotary shaker under the conditions described above, and harvested by filtration 10 days after inoculation to weigh the fresh cell mass. After the samples were dried in an air oven, the dry weight was also measured. Statistical analysis was done by Student's *t*-test. Number of replicates are shown in figure legends.

For the root growth test, the seeds of the T1 generation of an hMRP1 transformant (clone No. 3) were surface-sterilized with 1% sodium hypochloride for 15 min, washed three times with water, and placed on 0.8% agar plates containing a half-concentration of MS medium, 1.5% sucrose and various concentrations of cadmium. On the agar plate, 10 seeds of hMRP1 transformant and those of wild type were sown, and the plate was placed vertically under continuous light at 25 °C. The root length of the seedlings was measured 14 days after germination.

Measurement of cadmium

For the experiment using suspension cells, 100 μM cadmium chloride was added to 30 ml of modified MS medium, to which 1 g of fresh cells were inoculated and the cells were cultured at 25 °C under continuous light for 12 days. Aliquots (1 ml each) of the medium were sampled in the time-course experiment, and these were subjected to atomic absorption analyses with AA6300 (Shimadzu) after dilution with water according to the remaining concentration.

In the cadmium-tolerance experiment with regenerated plantlets, plantlets of ca. 10 cm in height were inoculated in 5 ml of modified MS medium containing 100 μM cadmium chloride, and then cultured at 25 °C under continuous light for 20 days. Medium samples were obtained (1.4 ml each) in the time-course experiment over 10 days, and these were then diluted with water and subjected to atomic absorption analyses. After cultivation for 20 days, the plantlets were divided into two parts, aerial part and underground part, and homogenized with 3 ml of distilled water, and the cadmium concentration of the extract was measured.

Measurement of chlorophyll content

Callus tissues (100 mg fresh weight) were collected at 7 days for measurement of chlorophyll content. The callus tissues were extracted with 80% (v/v) acetone–water in the dark at 4 °C overnight. The amount of chlorophyll was estimated according to the method by Porra (Porra *et al.*, 1989).

Results

Expression of hMRP1 *in tobacco cells*

The expression construct, pBI-El2-MRP1, is designed for the constitutive expression of a foreign gene by the enhancer-equipped CaMV35S promoter, which is ubiquitously very active in most cell types of the plant body, and the hygromycin-resistance gene is located at the right border sequence of the T-DNA (Figure 1A). With this binary vector, the full-length cDNA of hMRP1 was introduced into tobacco to obtain stable transformants. Twenty hygromycin-resistant

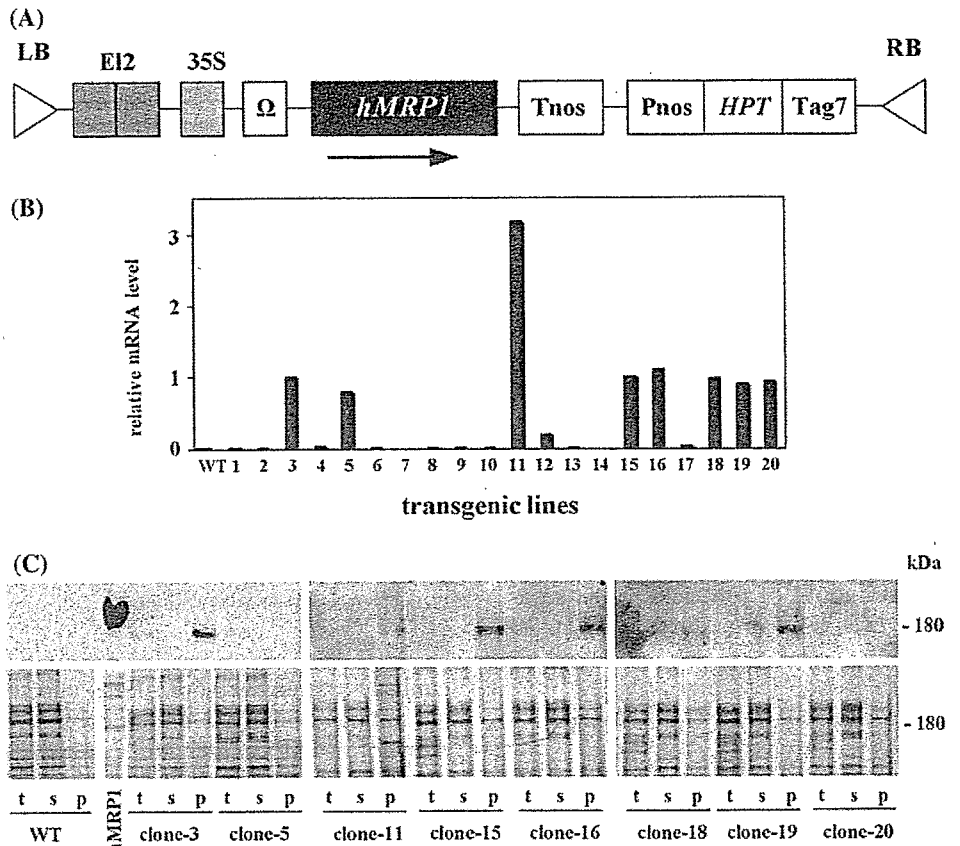

Figure 1. (A) Expression construct of human MRP1 for plant. The binary vector is a pBin19 derivative, which contains an expression cassette for plant cell and a selection marker, hygromycin-resistance gene (*HPT*) between the right and left borders. El2 is an enhancer sequence in tandem to increase the CaMV 35S promoter activity for constitutive expression of a foreign gene, under which the full-length cDNA of *hMRP* is subcloned, and the terminator is from nopalin synthase. For bacterial selection, kanamycin is used. (B) Northern analysis of kanamycin-resistant tobacco plants. Total RNA samples prepared from kanamycin-resistant tobacco leaves were probed with a *Bam* HI/*Eco* RI fragment of *hMRP1* cDNA (3.0 kb). 18S rDNA was used as a load control. The mRNA level relative to rRNA is shown to compare the accumulation levels of *hMRP1* mRNA among these clones. The mRNA level observed in clone No. 3 (MRP1-3) is defined to be one. (C) Western blot analysis of *hMRP1*-transgenic tobacco plants. Transgenic tobacco lines in which high levels of *hMRP1* mRNA accumulation were observed were used in the experiments. Total protein extracted from the leaves (7000×*g* sup; 't'), soluble fractions (100 000×*g* sup; 's') and microsomal fractions (100 000×*g* ppt; 'p') were separated by SDS-PAGE, and blotted for western analysis. Thirty microgram protein was loaded in each lane. Top panels show the accumulation of *hMRP1* protein in transgenic tobacco plants detected with the *hMRP1*-specific antibody MRPr1. Bottom panels show the separation pattern monitored with CBB staining. *hMRP1*-expressing membrane fraction of COS cells and wild type tobacco plants were used as positive and negative controls, respectively.

clones were analyzed to detect the accumulation of *hmrp1* mRNA by northern blot hybridization (Figure 1B). Out of 20 independent clones, *hmrp1* mRNA was detected in 9. The highest level of mRNA was observed in clone No. 11, and the other 8 clones showed a moderate expression of *hmrp1*, whereas there was no detectable band in the wild type tobacco used as a negative control, indicating that endogenous MRP molecules of tobacco did not cross-hybridize to the *hmrp1* probe. In hybridization, however, two bands were detected in some positive clones (data not shown), where the larger band corresponded to the full-size mRNA from migration.

Using eight transgenic tobacco clones (clone Nos. 3, 5, 11, 15, 16, 18, 19, and 20) that showed the accumulation of *hmrp1* mRNA, accumulation of its protein was detected by western blot with the

hMRP1-specific monoclonal antibody MRPr1 (Figure 1C). Total protein extracted from fresh leaves of these transformants, the microsomal fraction as well as the supernatant, was separated with SDS-PAGE and immunodetected with MRPr1. hMRP1 protein could be detected in six of these clones, and the highest expression was observed in clone No. 3. As expected, the hMRP1 protein was localized in the microsomal fraction, although the hMRP1 expressed in tobacco showed slightly smaller migration than that expressed in COS cells. According to previous reports, the hMRP1 protein migrated at ca. 190 kDa, whereas the heterologously expressed hMRP was detected at ca. 180 kDa, as shown in Figure 1C, which was still larger than the theoretical molecular weight (171 kDa) calculated from its amino acid sequence. This is probably due to the difference in the glycosidation state of hMRP1 in different organisms. The amount of protein and the separation pattern was also monitored with CBB staining.

Subcellular localization of hMRP1 *in tobacco*

The subcellular localization of transgenic hMRP in tobacco cells was analyzed by a conventional sucrose density gradient experiment. As shown in the non-linear density gradient centrifugation experiment (Figure 2), the hMRP1-rich fractions coincided with those of a vacuolar membrane protein marker, H^+-PPase (AVP1) (Mitsuda *et al.*, 2001), whereas the plasma membrane protein marker, H^+-ATPase (W1D) (Morsomme *et al.*, 1998), and the ER marker, luminal binding protein (BiP) (Shitan *et al.*, 2003), were fractionated in different patterns. We carried out linear sucrose gradient fractionation, which also supported the same conclusion that hMRP1 was localized on the vacuolar membrane of transgenic tobacco cells (data not shown).

Drug resistance of hMRP1*-expressing tobacco*

We surveyed the drug resistance of hMRP1-expressing tobaccos in comparison to the wild type, and found that the transgenic lines showed an apparent resistance against cadmium and daunorubicin. The former is a metal ion that can be transported by hMRP1 as well as by other MRP members in the form of a glutathione conjugate (Ishikawa *et al.*, 1996) and the latter is an anthracycline-type antibiotic, a typical substrate of hMRP1. Clone No. 3, which showed the highest hMRP1 expression at the protein level among the transgenic clones obtained, exhibited the highest resistance to these xenobiotics (Figure 3A, B for cadmium, Figure 3C, D for daunorubicin).

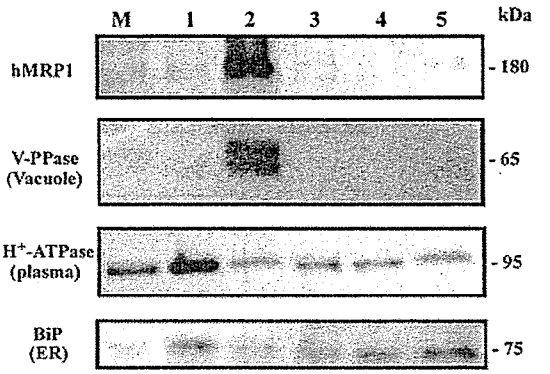

Figure 2. Subcellular localization of hMRP1 expressed in tobacco cells. The microsomal fraction of transgenic tobacco leaves was fractionated by centrifugation with a non-linear sucrose density gradient of 20% (fraction 1) to 60% (fraction 6). Each fraction was loaded on SDS-PAGE and blotted onto a PVDF membrane. Western blotting was carried out with monoclonal antibody against hMRP-1 (top), vacuolar pyrophosphatase (V-PPase, middle), and P-H^+ ATPase as a marker for plasma membrane (bottom).

Since hMRP1 can partially complement the cadmium resistance in yeast YCF1 (yeast cadmium factor-1) mutant (Tommasini *et al.*, 1996), it may be expected to function as a cadmium transporter in plant cells, although the resistance conferred was only moderate according to the fresh weight basis. In the presence of cadmium, the wild type tobacco cells appeared to be bleached due to the serious damage of chlorophyll, whereas the transgenic cells remained green even in the presence of 100 μM cadmium. Actually, the chlorophyll content in hMRP1-expressing plant (77.6 ± 10.3 μg/g fresh wt) hardly decreased (2.6%) by the cadmium treatment, whereas that in wild type plant (96.5 ± 13.4 μg/g fresh wt) decreased 17.8%, which was significant in the statistical analysis ($p < 0.05$). Daunorubicin inhibited the growth of cultured tobacco cells of wild type, while hMRP1-expressing tobacco cells showed clear resistance to this cytotoxic compound at concentrations of between 30 and

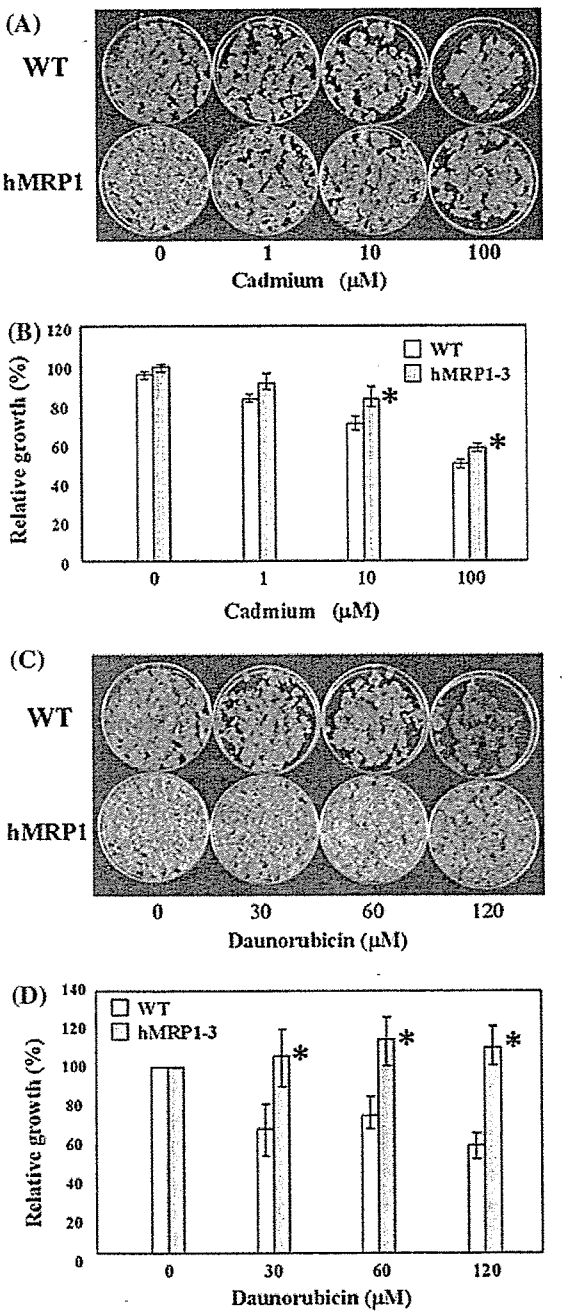

Figure 3. Drug resistance of hMRP1-transgenic tobacco cultured cells. (A) Cultured tobacco cells treated with 0–100 μM cadmium for 10 days. Wild type tobacco cells used as the negative control are shown in the upper row, and transgenic tobacco expressing hMRP1 is shown in the lower row. (B) Relative growth of cultured cells. Fresh cell mass relative to that in the cadmium-free medium is defined as 100% (8.8 g for wild type and 8.7 g for transgenic cells). Standard deviation is calculated with three replicates. (C) Cultured tobacco cells treated with 0–120 μM daunorubicin for 10 days. Wild type tobacco cells used as a negative control are shown in the upper row, and transgenic tobacco expressing hMRP1 is shown in the lower row. (D) Relative growth of the cultured cells. Fresh cell mass relative to that in the daunorubicin-free medium is defined as 100%. Standard deviation is calculated with three replicates and asterisk indicates statistically significant difference ($p < 0.05$).

120 μM (Figure 3C, D), which did not affect cell growth in the transgenic line.

In the experiment with the administration of daunorubicin, which is an orange compound, the color disappeared from the medium more efficiently in the transgenic clones than in the wild type cells. Figure 4 shows a comparison of the decrease in the orange color of daunorubicin among independent transgenic clones, which expressed hMRP1 polypeptide at different levels (see Figure 1C). According to the expression level, the rate of the decrease in orange color remaining in the medium was prominent, which was also confirmed by HPLC analysis (data not shown). This observation is consistent with the fact that the hMRP polypeptide is localized at the vacuolar membrane in tobacco as shown in Figure 2; i.e., this ABC transporter functions as a drug efflux pump to transport daunorubicin from cytosol into the vacuolar lumen.

Next, we carried out a time-course experiment on cadmium uptake from the medium by cultured cells (wild type and hMRP1 transgenic clone No. 3). Figure 5 shows that cadmium in the medium decreased both in wild type and hMRP-expressing

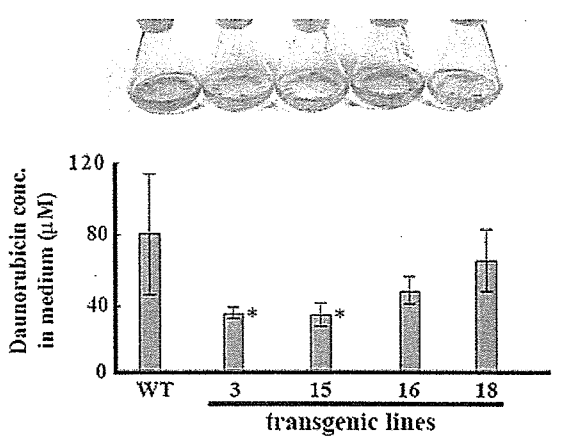

Figure 4. Comparison of daunorubicin remaining in the medium among hMRP1 transgenic clones. These clones are the same as those that were used for the western blot shown in Figure 2. Daunorubicin (120 μM) was added upon inoculation of the cells, which were cultured for 3 days in the dark. A higher level of hMRP1 expression in the tobacco clones was associated with a more prominent decrease in orange color due to daunorubicin. Wild type tobacco is the negative control. From left to right, the flasks show wild type, and clone Nos. 3, 15, 16, and 18. Standard deviation is calculated with three replicates and asterisk indicates statistically significant difference ($p < 0.05$).

tobacco cell cultures, whereas the decrease in transgenic cell cultures seemed to be faster than that in wild type cell cultures, particularly at the beginning of the culture period. We measured then the cellular content of cadmium for both cultures. The cellular content of cadmium was calculated to be 71 ± 11 μM for both cultured cells at day 7, and no statistical difference between transgenic and wild type cell cultures was observed. The cadmium amount recovered from the cells was much less than the cadmium disappeared from the medium. This might be because cadmium in the medium formed insoluble complex in the medium or on the cell wall. The cellular content of cadmium is, however, toxic level for plant cell, whereas the hMRP1-expressing cells showed resistance against cadmium, which suggested that this ABC transporter contributed to sequestrate the heavy metal in the vacuolar lumen.

The cadmium-resistance of transgenic tobacco expressing hMRP1 was also tested with regenerated tobacco plants. Figure 6 illustrates the cadmium-tolerance of seedlings of hMRP1-transgenic clones, as evaluated by their root length. The transgenic lines (clone No. 3) showed higher tolerance to cadmium in the medium at a concentration range between 120 and 240 μM. The roots of wild type tobacco tended to show branching of the root tissue on plates containing cadmium at the concentration range tested, whereas those of hMRP-transgenic tobacco seedlings grew without showing root branching, as on the control medium. This branching may be caused by the suppression of growth at the root tip due to the toxicity of cadmium, which might induce new root generation.

Next, we tested the cadmium-tolerance of hMRP1-expressing clone using 1-month-old plants of the T3 generation. Figure 7A shows tobacco plants grown hydroponically in the presence of cadmium (100 μM). After 20 days, the hMRP1-transgenic tobacco was growing continuously and appeared green, whereas the growth of wild type tobacco was strongly suppressed and leaves had started to turn yellow. The cadmium concentration remaining in medium was also measured: it decreased almost linearly to 20–30% of the initial concentration in 2.5 days, and then gradually decreased to 10–20% in 10 days (Figure 7B). The curves for the decrease in cadmium were nearly identical for both wild type and transgenic plants.

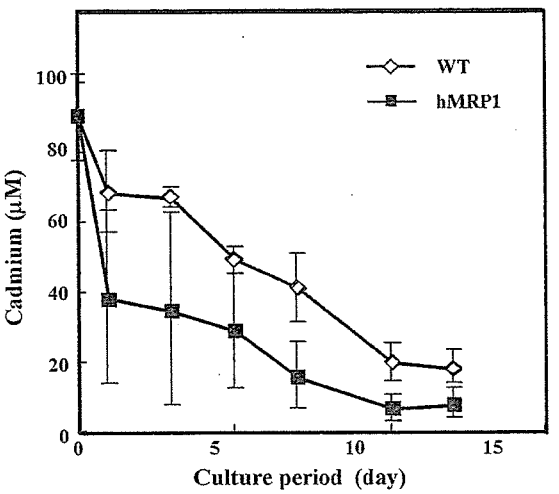

Figure 5. Time-course of cadmium uptake from the culture medium by cultured cells of wild type and hMRP1-transgenic tobacco. The initial concentration of cadmium in the medium is 100 μM, to which wild type or hMRP1-expressing tobacco cells are inoculated. The cadmium concentration in the culture medium was monitored by atomic absorption. Standard deviation is calculated with five replicates.

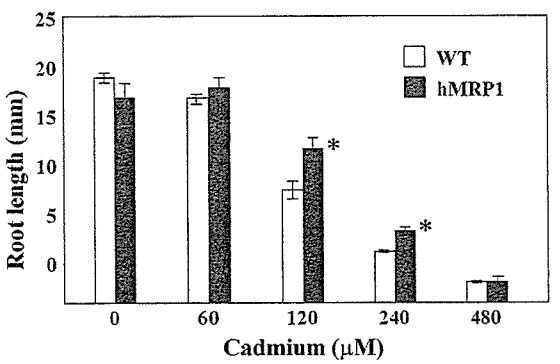

Figure 6. Effect of cadmium on the root growth of hMRP1-transgenic tobacco. Root length (mm) of wild type and hMRP1-transgenic tobacco seedlings in the presence of 0–480 μM cadmium. Standard deviation is calculated with 20 seedlings per column and asterisk indicates statistically significant difference ($p < 0.05$).

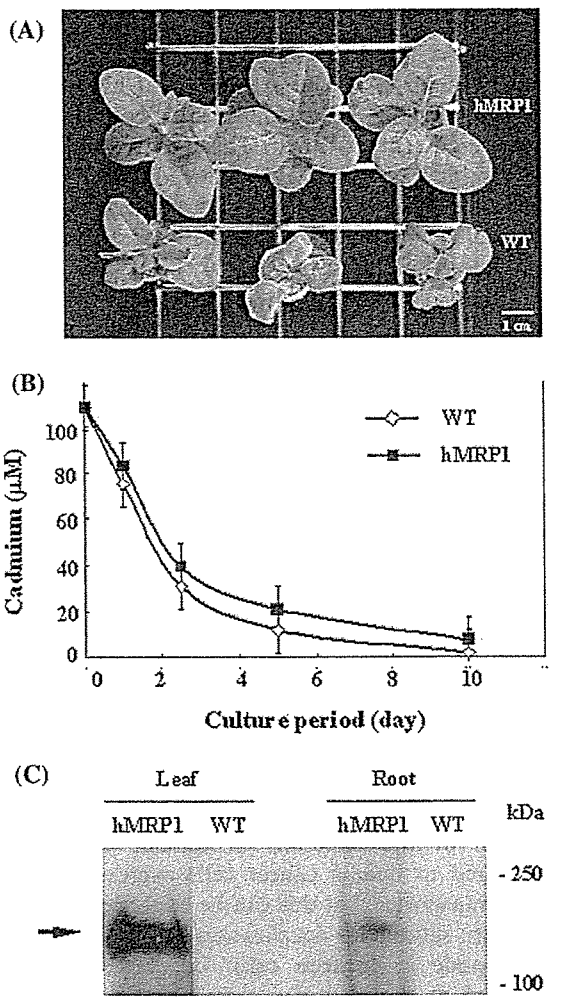

Figure 7. Cadmium-resistance of hMRP1-transgenic tobacco plants. (A) hMRP1-transgenic tobacco (upper) and wild type tobacco (under) after treatment with 100 μM cadmium for 20 days. (B) Time-course of cadmium uptake from the culture medium by cultured cells of wild type and hMRP1-transgenic tobacco for 10 days. Standard deviation is calculated with three seedlings per point. (C) The expression of hMRP1 in root was analyzed by western blot analysis. The leaf of hMRP1-transgenic tobacco was used as a positive control, and wild type was used as a negative control.

To determine where cadmium was accumulated in the plant body, the cadmium concentration was measured in the aerial part and the underground part. As a result, almost same amount of cadmium was detected in leaf (5.4–9.3 μg/g fresh wt) and root tissues (21.1–27.5 μg/g fresh wt) for both transgenic and wild type plants, where no statistical significance was observed, and the ratio of cadmium in the aerial part to that in the underground part was not significantly affected by hMRP1 expression, i. e., transgenic plants $29 \pm 18\%$ and wild type plants $23 \pm 8\%$. These data suggest that the absorption of cadmium did not seem to be influenced by hMRP1. To clarify

this possibility, we performed western analysis of root tissue for comparison with the results in leaves (Figure 7C). The expression of hMRP1 in the root of clone 3 was found to be considerably lower than that in leaves. This observation may explain, at least partly, why the cadmium uptake was not distinguishable but the tolerance was apparently different in leaves between transgenic and wild type plants.

We tried to analyze the tolerance of hMRP1-tobacco plants to daunorubicin, which was prominent in suspension cultures, but the experiment was not feasible because of the instability of this compound under light irradiation, which was necessary for plant growth.

Discussion

This study has demonstrated that human MRP1 is stably expressed in tobacco cells, and conferred tolerance to a heavy metal and an organic drug. This is a novel approach for 'transport engineering' by the heterologous expression of mammalian ABC transporter (Zaman *et al.*, 1994) in plants. Transgenic cell lines absorb daunorubicin from the medium more efficiently. This is an apparent reversal of the direction of cellular transport compared to human cancer cells expressing this ABC transporter, but this can be explained by the different subcellular localization of the hMRP1 polypeptide in tobacco cells; i.e., hMRP1 was localized at the vacuolar membrane. From the viewpoint of cytosol, the efflux of drugs to the apoplast across the plasma membrane and that to the vacuolar lumen across tonoplast membrane are actually equivalent events in terms of the exclusion of xenobiotics (Lu *et al.*, 1997; Liu *et al.*, 2001). In case of cadmium, decrease in cadmium from the medium did not match that recovered from the cultured cells and from regenerated plant tissues, which may be because the formation of insoluble complex of cadmium in the medium or on the cell walls. The same level of cadmium was detected in hMRP1-tobacco as well as in wild type, but the transgenic plant revealed clear tolerance to this toxic heavy metal. This strongly suggests that hMRP1 contributed to sequestrate cadmium in vacuoles.

It is not clear why hMRP1 is localized at tonoplast in tobacco cells, while its localization in animal cells is plasma membrane (Deeley and Cole, 1997). Recent publication has shown that hMRP1 is also expressed in intracellular vesicle compartments of animal cells where it actively sequesters drugs away from the nucleus (Rajagopal and Simon, 2003), suggesting the similar targeting of hMRP1 to lytic organelles, as lysosome in animal cells and vacuole in plant cells. Although the hMRP1 polypeptide does not contain a typical targeting signal for particular organelles, we cannot exclude the possibility that there might be a yet unknown signal sequence in hMRP polypeptide, which is functioning as a vacuolar targeting signal in tobacco cells. The differential localization of an ABC protein in a heterologous host was also reported for YCF1, which was localized in both tonoplast and plasma membrane when expressed in *Arabidopsis*, while YCF1 is a vacuolar protein in yeast (Song *et al.*, 2003).

In human cells, hMRP1 is glycosylated and therefore shows a molecular weight of ca. 190 kDa in SDS-PAGE (Deeley and Cole, 1997), whereas the recombinant hMRP1 expressed in yeast appears at ca. 170 kDa, which is nearly the theoretical size of its deduced amino acid sequence (Tommasini *et al.*, 1996). The hMRP1 polypeptide expressed in tobacco migrated almost at ca. 180 kDa in western blot analyses, indicating that it is glycosylated but in a different manner from mammals. However, this glycosidation is not thought to influence the basic transport function of hMRP1 to confer multidrug resistance to the cells (Di Virgilio *et al.*, 1988).

While members of the MRP family are also widely found in plant cells (Martinoia *et al.*, 2000), to the best of our knowledge, no endogenous members of the MRP family in tobacco have yet been analyzed. In *Arabidopsis*, however, MRP is one of the most intensively studied ABC transporter subfamilies. AtMRP1 and AtMRP2 seem to be mostly involved in detoxification by transporting glutathione conjugates of several organic compounds (Lu *et al.*, 1997; Liu *et al.*, 2001), and a member AtMRP3 was also reported to confer cadmium tolerance (Tommasini *et al.*, 1998). Subcellular localization of *Arabidopsis* MRPs, such as AtMRP1 and AtMRP2, was reported to be at vacuolar membrane. Endogenous members of the MRP family in tobacco cells may be involved in sequestrating cadmium or daunorubicin in vacuoles, but perhaps the transport efficiency is not

sufficient or the expression level might be too low to detoxify high concentrations of these xenobiotics, and thus there is a clear difference in the tolerance between wild type and hMRP1-tobacco lines.

Regarding the substrate specificity, hMRP1-expressing tobacco gave interesting results. Etoposide, an anticancer lignan, is one of the most preferable substrates for hMRP1 (Jedlitschky *et al.*, 1996), while hMRP1-expressing tobacco cells did not show significant tolerance to this compound compared to the wild type (data not shown). One explanation for this phenomenon is that etoposide is first conjugated with glucuronic acid to be recognized by hMRP1, and then transported (Renes *et al.*, 1999), whereas glucuronide does not play a major role in detoxification in plants, and glucosidation and glutathione as well as malate conjugates play pivotal roles (Theodoulou, 2000). In fact, hMRP1-expressing tobacco also showed tolerance to the herbicide alachlor and acifluorfen, which are detoxified by conjugation with glutathione (supplemental information). Another possible explanation for the difference in substrate specificity is a difference in the lipid composition of membranes. It has been reported that the lipid composition greatly affects the transport function of ABC transporters (Saeki *et al.*, 1992), and these differences between tobacco tonoplast and human plasma membrane may also explain why tolerance to etoposide was not observed in hMRP1-expressing tobacco.

The MRP1-transgenic tobacco may offer a new possibility to use ABC transporter gene for phytoremediation to clean the soil or water environment. In conventional phytoremediation, the production of a chelator molecule, e.g. phytochelatine, is a popular strategy at heavy metal tolerance including cadmium (Zhu *et al.*, 1999), or metabolic enzymes are introduced to decompose organic pollutants (Doty *et al.*, 2000). Such remediators can be applied to simple pollution either by heavy metals or a few organic pollutants, but are not suitable for complex pollution consisting of mixtures, which is often the actual environmental problem in the real situation. It is possible that plant remediators expressing ABC transporters may be used for such complex pollution because some members show broad substrate specificity for many organic compounds of divergent structures and also for some inorganic compounds.

However, there are still some problems to be solved before any actual application; e.g. ABC transporters cannot always be stably expressed in heterologous host plants. Using the same construct presented in this study, many transgenic lines were prepared in potato, in which hMRP1 mRNA is highly expressed, while the level of hMRP1 protein was below the limit of detection (data not shown). Similarly, we tried to express human MDR1 in tobacco, but no reasonable accumulation of MDR1 polypeptide was observed even though high expression was seen at the mRNA level. Since it is not possible to regulate the compatibility of gene and host organism at the current technology level, many combinations of ABC transporter genes and host plants should be tried.

The transgenic tobacco established in this study has the potential to keep growing in cadmium-polluted soil due to the clear tolerance, and thus keep absorbing the heavy metal. The further introduction of metal chelator genes to this MRP-expressing plant may provide a 'super remediator plant' that is capable of extracting heavy metal pollutants from the soil more effectively and safely accumulating them at a higher level in the plant mass.

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