Regulation of Function by Dimerization through the Amino-terminal Membrane-spanning Domain of Human ABCC1/MRP1*

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Overexpression of some ATP-binding cassette (ABC) membrane transporters such as ABCB1/P-glycoprotein/MDR1 and ABCC1/MRP1 causes multidrug resistance in cancer chemotherapy. It has been thought that half-ABC transporters with one nucleotide-binding domain and one membrane-spanning domain (MSD) likely work as dimers, whereas full-length transporters with two nucleotide-binding domains and two or three MSDs function as monomers. In this study, we examined the oligomeric status of the human full-length ABC transporter ABCC1/MRP1 using several biochemical approaches. We found 1) that it is a homodimer, 2) that the dimerization domain is located in the amino-terminal MSD0L0 (where L0 is loop 0) region, and 3) that MSD0L0 has a dominant-negative function when coexpressed with wild-type ABCC1/MRP1. These findings suggest that ABCC1/ MRP1 may exist as a dimer and that MSD0L0 likely plays some structural and regulatory functions. It is also tempting to propose that the MSD0L0-mediated dimerization may be targeted for therapeutic development to sensitize ABCC1/ MRP1-mediated drug resistance in cancer chemotherapy.

Multidrug resistance is a serious problem in successful cancer chemotherapy. Studies using model cell lines have suggested that overexpression of some ATP-binding cassette (ABC) membrane transporters such as P-glycoprotein (ABCB1/MDR1) and MRP1 (multidrug resistance-associated protein 1; ABCC1) causes multidrug resistance. These ABC transporters actively efflux anticancer drugs out of cells and thus effectively reduce the intracellular accumulation and cytotoxicity of these drugs (1–4). In humans alone, the ABC transporters comprise a 49-member superfamily, which is divided into seven subfamilies (ABCA–G; nutrigene.4t.com/humanabc.htm), although two of the members lack transmembrane domains and do not qualify as transporters by themselves.

Human ABCC1/ MRP1 (referred to hereafter as ABCC1) is 1 of the 13 members of the human ABC subfamily. ABCC1 has been demonstrated to mediate ATP-dependent cellular efflux of a wide variety of anticancer drugs and xenobiotics and a broad spectrum of organic anions, including GSSG and GSH as well as anionic conjugates of GSH, glucuronide, and sulfate (3, 5). Endogenous organic anion substrates of ABCC1 include cysteinyl leukotriene C4 (LTC4) and conjugated estrogens β-estradiol 17-(β−d-glucuronide), estrone 3-sulfate, and dehydroepiandrosterone sulfate (6–10).

Unlike most of other human ABC transporters such as ABCB1/P-glycoprotein/MDR1, which contain a core structure of MSD1-NBD1-MSD2-NBD2 (Fig. 1), ABCC1, as well as ABCB2, ABCB3, ABCB6, and ABCB8–10, contains an additional MSD (MSD0) at the amino terminus that consists of five predicted transmembrane segments with a putative extracellular amino-terminal end (11–14). Although it has recently been argued that the amino-terminal end (33 amino acids) may form a U-shaped structure and function as a gate (15, 16), the functional role of MSD0 remains to be determined.

Half-ABC transporters with a single MSD and a single NBD have been thought to function as dimers (2). However, the human half-ABC transporter ABCC2 has been shown recently to exist mainly as a homododecamer (17), suggesting that it may function as a oligomer with 12 subunits. Previous studies using radiation inactivation of human erythrocytes (18) and electron microscopy imaging of purified ABCC1 (19) have suggested that ABCC1 may be a dimer. In this study, we examined the oligomeric status of human ABCC1 using multiple approaches, including perfluorooctanoic acid (PFO)-PAGE, nondenaturing PAGE, gel filtration chromatography, sucrose density gradient sedimentation, chemical cross-linking, and co-immunoprecipitation. We demonstrate that human ABCC1 is a homodimer and that MSD0L0 is essential and sufficient for homodimerization of human ABCC1. Although coexpression of MSD0L0 inhibits the LTC4 transport activity of full-length

* The abbreviations used are: ABC, ATP-binding cassette; LTC4, leukotriene C4; MSD, membrane-spanning domain; NBD, nucleotide-binding domain; PFO, perfluorooctanoic acid; L0, loop 0; DSP, dithiobis(succinimidyl propionate); HA, hemagglutinin; PVDF, polyvinylidene difluoride; DTT, dithiothreitol.

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**Materials**—PFO and dithiobis(succinimidyl propionate) (DSP) were purchased from Oakwood Products, Inc., and Pierce, respectively. Monoclonal antibody MRPr1 and anti-he-magglutinin (HA) antibody were from Kamiya Biomedical Co. and Covance Inc., respectively. Anti-FLAG antibody M2, horseradish peroxidase-conjugated goat anti-mouse IgG and rabbit anti-rat IgG, β-galactosidase, Triton X-100, and LTC₄ were from Sigma. Radioactive [³H]LTC₄ was purchased from PerkinElmer Life Sciences. Polyvinylidine difluoride (PVDF) membranes, concentrated protein assay dye reagents, and precast polyacrylamide gradient gels were from Bio-Rad. Lipofectamine, G418, and cell culture media and reagents were obtained from Invitrogen. A Superose 6 HR column, thryoglobulin, ferritin, catalase, bovine serum albumin, and an enhanced chemiluminescence (ECL) system were from Amersham Biosciences. Laminin and protein G-agarose were from BD Biosciences and Santa Cruz Biotechnology, Inc., respectively. All other reagents were molecular biology grade and were purchased from Sigma or Fisher.

**EXPERIMENTAL PROCEDURES**

**Engineering Human ABCC1 Constructs**—pcDNA3.1(+)−MRP1WT encoding human wild-type ABCC1 was constructed previously (20). The constructs encoding full-length ABCC1 with a FLAG or HA tag at its carboxyl terminus were engineered by amplifying a 754- or 763-bp fragment of ABCC1 using a forward primer (nucleotides 4012–4029 of human ABCC1 cDNA) with an EcoRI site (underlined) (5′-GTTGAA-TTCCTCGGAAGCTAC-3′) and a reverse primer with a NotI site (underlined) and a FLAG tag (italic) (5′-CCGCGGCGGCTCA-CTTTGATCTGTCGCTCTTGTGAGCTACAAAGCGGCGG- TCTTTGGC) or an HA tag (italic) (5′-CCGCGGCGGCTCA-GAGGTCAGCATATATGACAACTATACGGAATACACCC- AAAGCAGCGCTTCTTTGGC). The PCR products were then digested with EcoRI and NotI to generate a 746-bp (FLAG) or 755-bp (HA) fragment, which was subsequently cloned into pcDNA3.1(+)−MRP1WT digested with EcoRI and NotI, resulting in pcDNA3.1(+)−ABCC1F-FLAG or pcDNA3.1(+)−ABCC1F-HA. To generate the human carboxyl-terminal core structure of ABCC1 with a FLAG tag at its carboxyl terminus, the 746-bp fragment from the PCR was inserted into previously constructed pcDNA3.1(+)−MRP1CORE (20) digested with EcoRI and NotI, resulting in pcDNA3.1(+)−ABCC1CORE-FLAG. To generate construct pcDNA3.1(+)−ABCC1281N encoding the first 281 amino acids of human ABCC1, pcDNA3.1(+)−MRP1WT (20) was digested with BamHI and NotI and blunted with Klenow fragment. The DNA fragments were then self-ligated, resulting in pcDNA3.1(+)−ABCC1281N. Translation of ABCC1281N terminates at a stop codon in the vector, resulting in the addition of 9 amino acids (RPLESGPGV) following Asp²⁸¹ of human ABCC1, as described previously (21, 22). All constructs were confirmed by double-strand DNA sequencing.

**Cell Culture, Transfection, and Membrane Preparations**—HEK293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum in the presence of 100 units/ml penicillin and 100 μg/ml streptomycin. To establish stable ABCC1F-HA clones, 5 μg of pcDNA3.1(+)−ABCC1F-HA were transfected into HEK293 cells in 100-mm cell culture dishes using Lipofectamine according to the manufacturer’s instructions. Two days following transfection, 10% of the transfected cells were selected with 400 μg/ml G418 for 2 weeks. The G418-resistant cells were cloned using cloning cylinders and propagated for further experiments.

For transient transfection, 15 μg of pcDNA3.1(+)−ABCC1F-FLAG, pcDNA3.1(+)−ABCC1281N, or pcDNA3.1(+)−ABCC1CORE-FLAG were transfected into HEK293 cells that did or did not express ABCC1F-HA in 150-mm dishes using Lipofectamine according to the manufacturer’s instructions. Forty-eight hours after transfection, cells were harvested for preparation of cell lysates or membrane vesicles as described previously (20).

**PFO-PAGE**—Extractions of human ABCC1 from membranes with PFO and PFO-PAGE were performed as described previously (17) with minor modifications. Briefly, 5 μg of plasma membranes in 250 mM sucrose, 10 mM Tris (pH 7.4), and 150 mM NaCl were mixed with an equal volume of 2× PFO extraction sample buffer (100 mM Tris (pH 8.0), 20% (v/v) glycerol, 0.005% bromphenol blue, 200 mM dithiothreitol (DTT), and 0.25–8% PFO) or 2× SDS sample buffer (100 mM Tris-HCl (pH 6.8), 20% glycerol, 0.005% bromphenol blue, 200 mM DTT, and 4% SDS) and incubated at room temperature for 30 min.
followed by centrifugation at 11,000 \( \times g \) for 10 min to remove insoluble materials. The supernatants were loaded onto freshly prepared 7.5\% Tris/glycine-polyacrylamide gel without SDS. Electrophoresis was performed at 100 V at 4 °C using a running buffer containing 25 mM Tris (pH 8.5), 192 mM glycine, and 0.1\% PFO. The proteins were then transferred to PVDF membranes, and human ABCC1 was detected using monoclonal antibody MRPr1. The signal was detected using horseradish peroxidase-conjugated rabbit anti-rat secondary antibody and the ECL system.

**Nondenaturing PAGE**—Nondenaturing PAGE was performed as described previously (17). Briefly, ~10 \( \mu \)g of plasma membranes in 250 mM sucrose, 10 \( \mu \)M Tris (pH 7.4), and 150 mM NaCl were mixed with an equal volume of 2\( \times \) Triton X-100 extraction sample buffer (100 mM Tris (pH 8.0), 40\% glycerol, 0.005\% bromphenol blue, 2\% Triton X-100, and 200 mM DTT) or 2\( \times \) SDS sample buffer and incubated at room temperature for 30 min, followed by centrifugation at 11,000 \( \times g \) for 10 min to remove insoluble materials. The supernatants were then loaded onto a 4–15\% gradient Tris/glycine-polyacrylamide gel, and electrophoresis was performed at 80 V at room temperature with a running buffer containing 25 mM Tris (pH 8.3) and 192 mM glycine, followed by transfer to PVDF membranes for Western blot analysis as described above.

**Sucrose Density Gradient Sedimentation**—Plasma membranes (100 \( \mu \)g) were first extracted with 2\( \times \) sample buffer (100 mM Tris-HCl (pH 8.0), 200 mM DTT, and 1\% PFO or 2\% SDS) as described above. The extracts were then loaded onto a 10–30\% (w/v) continuous sucrose gradient containing 50 mM Tris-HCl (pH 7.4), 1 mM DTT, and 0.1\% PFO or SDS. Sedimentation was performed with a Beckman SW 41 rotor at 100,000 \( \times g \) for 18 h at 4 °C. Fractions (0.5 ml) were collected, followed by trichloroacetic acid precipitation, separation by SDS-PAGE, transfer to PVDF membranes, and detection of ABCC1 by Western blot analysis using monoclonal antibody MRPr1 as a probe. Protein markers (thyroglobulin (669 kDa), laminin (400 and 200 kDa), catalase (232 kDa), and bovine serum albumin (66 kDa)) were separated and fractionated under the same conditions and detected by Coomassie Blue staining of the SDS-polyacrylamide gel of each fraction.

**Gel Filtration Chromatography**—Gel filtration chromatography was performed using an ÄKTA purifier system with a Superose 6 HR column as described previously (17). Briefly, 100 \( \mu \)g of plasma membranes were first extracted with PFO or SDS in buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 100 mM DTT for 30 min at room temperature and then subjected to centrifugation at 11,000 \( \times g \) for 10 min. The supernatants were injected into the column equilibrated with elution buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM DTT, and 0.1\% PFO or SDS). Fractions (0.5 ml each) were collected, followed by trichloroacetic acid precipitation. The retention of human ABCC1 was determined by separation of each fraction by SDS-PAGE and transfer to PVDF membranes, followed by detection of ABCC1 by Western blot analysis. Protein markers (laminin (400 and 200 kDa), \( \beta \)-galactosidase (116 kDa), and bovine serum albumin (66 kDa)) were separated using the ÄKTA purifier system under the same conditions and detected using the UV detector of the ÄKTA purifier system.

**Chemical Cross-linking**—A stable HEK293 cell clone expressing human ABCC1 (20) in culture was washed three times with phosphate-buffered saline and then treated with different concentrations of DSP at room temperature for 30 min, followed by the addition of Tris (pH 7.4) to a final concentration of 20 mM and incubation for another 15 min to terminate the cross-linking reaction. Crude membranes were prepared as described previously (20), followed by separation by SDS-PAGE in the absence or presence of 100 mM DTT before transfer to PVDF membranes and Western blot analysis using monoclonal antibody MRPr1 as a probe.

**Immuno precipitation**—Immunoprecipitation was performed essentially as described previously (17, 23). Briefly, cells were washed three times with ice-cold phosphate-buffered saline and then lysed with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100). The cell lysate was cleared of insoluble materials by centrifugation and used for immunoprecipitation. For inhibition of glycosylation, tunicamycin treatment was performed prior to lysis preparation as described previously (20). For immunoprecipitation, cell lysates (1.5 mg) were first precleared by incubation with 1 \( \mu \)g of normal mouse IgG at 4 °C for 4 h, followed by the addition of 50 \( \mu \)l of protein G-agarose beads (50% slurry) and incubation at 4 °C for 3 h with rotation and centrifugation at 500 \( \times g \) for 5 min. The cleared supernatants were then incubated with 10 \( \mu \)g of anti-FLAG or anti-HA antibody at 4 °C with rotation for 2 h before mixing with 50 \( \mu \)l of protein G-agarose slurry. The mixtures were further incubated at 4 °C for 3 h with rotation, followed by centrifugation to collect precipitates, which were then washed five times with lysis buffer and used for Western blotting.

**\[^{3}H\]LT C\(_{4}\) Transport Assay**—ATP-dependent transport of \[^{3}H\]LT C\(_{4}\) into inside-out plasma membrane vesicles was measured using a rapid filtration method as described previously (20, 24). Briefly, 2 \( \mu \)g of membrane vesicles were incubated at 25 °C for 2 min in 25 \( \mu \)l of transport buffer (50 mM Tris-HCl, 250 mM sucrose, and 0.02% sodium azide (pH 7.4)) containing 4 mM ATP or AMP, 10 mM MgCl\(_{2}\), 100 \( \mu \)g/ml creatine kinase, 10 mM creatine phosphate, and 50 nM \[^{3}H\]LT C\(_{4}\) (0.01 \( \mu \)Ci) and mixed with 1 ml of ice-cold transport buffer, followed by filtration under vacuum through a glass-fiber filter (type GF/B, Whatman). Filters were immediately washed twice with 5 ml of ice-cold transport buffer and then dried before measurement of radioactivity by scintillation counting.

**RESULTS**

**PFO Extraction and PFO-PAGE Analyses of Human ABCC1**—To investigate the oligomeric status of human ABCC1, we first employed PFO-PAGE. PFO is a mild ionic detergent that does not break the noncovalent interactions between protein subunits of an oligomer at appropriate concentrations and therefore permits extraction and determination of the oligomeric status of membrane proteins by PFO-PAGE. This method has been used successfully to study the oligomeric status of several membrane proteins (17, 25–28).

We first tested the optimal PFO concentration to extract human ABCC1 from plasma membranes of HEK293 cells expressing ectopic human ABCC1 (20) using SDS-PAGE and
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Western blot analysis. As shown in Fig. 2A, PFO at concentrations of 0.5% and above can effectively extract human ABCC1 from membranes. When the PFO-extracted human ABCC1 proteins were separated by PFO-PAGE, most migrated with an estimated molecular mass of ~569 kDa at low concentrations (e.g. 0.5%) of PFO (Fig. 2B). The SDS-extracted ABCC1 proteins migrated with an estimated molecular mass of ~258 kDa and were assumed to be monomers (Fig. 2B and Table 1). Thus, most of the ABCC1 proteins extracted with 0.5% PFO migrated likely as dimers (Table 1). However, more monomeric ABCC1 proteins were apparent when higher concentrations (e.g. 4%) of PFO were used for extraction (Fig. 2B), suggesting that higher concentrations of PFO can dissociate ABCC1 into monomers.

To determine that the formation of dimeric ABCC1 was not due to the use of PFO, the nonionic detergent Triton X-100 was used for extraction, followed by nondenaturing PAGE and Western blot analysis. ABCC1 extracted with SDS was used as a control. As shown in Fig. 2C, Triton X-100-extracted ABCC1 migrated with an apparent molecular mass of 534 kDa, whereas SDS-extracted ABCC1 migrated with an apparent molecular mass of 289 kDa. Assuming that ABCC1 extracted with SDS migrated as a monomer, we conclude that the ~534-kDa ABCC1 protein extracted with Triton X-100 is likely a dimer (Table 1).

Analysis of Human ABCC1 by Sucrose Density Gradient Sedimentation—Sucrose density gradient sedimentation was also used to determine the size of human ABCC1. ABCC1 was first extracted from membranes with PFO or SDS as a control and then subjected to sucrose density gradient sedimentation, followed by fractionation, trichloroacetic acid precipitation, SDS-PAGE, and Western blot analysis as described under “Experimental Procedures.” As shown in Fig. 3 (A and B), ABCC1 extracted with PFO was detected between fractions 7 and 11 with a peak in fraction 9, with an estimated molecular mass of 319 kDa. On the other hand, ABCC1 extracted with SDS was detected between fractions 15 and 16 (Fig. 3C), with an estimated average molecular mass of 132 kDa (Fig. 3D). Thus, the majority of ABCC1 proteins extracted with PFO likely exist as dimers, assuming that ABCC1 proteins extracted with SDS behave as monomers (Table 1).

Analysis of Human ABCC1 by Gel Filtration Chromatography—We next performed gel filtration chromatography separation of ABCC1 extracted with PFO or SDS as a control using a Superose 6 HR column, followed by trichloroacetic acid precipitation and Western blot analysis. As shown in Fig. 4 (A and B), human ABCC1 extracted with PFO eluted in fractions with retentions of 8–14 ml. The two peak fractions with retentions of 10 and 12.5 ml had estimated molecular masses of 412 and 202 kDa, respectively. On the other hand, ABCC1 extracted with SDS eluted between fractions of 9.5–11 ml, with an estimated molecular mass of 189 kDa at the peak (Fig. 4, C and D). Thus, the two peak fractions of ABCC1 extracted with PFO are likely dimeric and monomeric forms, respectively (Table 1).

Chemical Cross-linking of Dimeric ABCC1 in Live Cells—The above experiments showed that human ABCC1 extracted with PFO or Triton X-100 has the apparent molecular mass of dimeric ABCC1. However, isolated membranes were used in all of these experiments, and a mild detergent had to be used to preserve the noncovalent protein-protein interactions. To determine whether dimeric ABCC1 exists in live cells without the use of detergent and membrane isolation, we performed chemical cross-linking with DSP, a thiol-cleavable, lipophilic, and bifunctional cross-linking reagent with a spacer arm length of 12 Å. For this purpose, live HEK293 cells expressing human ABCC1 were first treated with or without different concentrations of DSP, followed by

| Method of separation | Molecular mass | No. of subunits | Oligomeric state |
|----------------------|----------------|-----------------|-----------------|
| PFO-PAGE             |                |                 |                 |
| PFO                  | 569            | 2.2             | Dimer           |
| PFO                  | 258            | 1.0             | Monomer         |
| SDS                  | 258            | 1.0             | Monomer         |
| Nondenaturing PAGE   |                |                 |                 |
| Triton X-100         | 534            | 1.9             | Dimer           |
| SDS                  | 289            | 1.0             | Monomer         |
| Gradient sedimentation |                |                 |                 |
| PFO                  | 319            | 2.4             | Dimer           |
| SDS                  | 132            | 1.0             | Monomer         |
| Gel filtration       |                |                 |                 |
| PFO                  | 412            | 2.2             | Dimer           |
| PFO                  | 202            | 1.1             | Monomer         |
| SDS                  | 189            | 1.0             | Monomer         |

* The number of ABCC1 subunits was calculated based on the apparent molecular mass measured using each method in the presence of SDS, under which condition ABCC1 was assumed to migrate as a monomer. Note that the apparent molecular mass of ABCC1 in SDS varied as determined using different methods of separation. However, the ratio between the molecular mass determined in the presence of PFO or Triton X-100 and that determined in the presence of SDS was always close to 2.

**Table 1**

Summary of the apparent molecular masses and oligomeric states of human ABCC1

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solubilization of membranes and separation by SDS-PAGE in the absence or presence of 100 mM DTT for Western blot analysis. As shown in Fig. 5A, ABCC1 without DSP treatment had an apparent molecular mass of 168 kDa (lanes 1 and 2). Following DSP cross-linking, we detected a protein of 314 kDa (lanes 3, 5, and 7), corresponding to the size of dimeric ABCC1 (Table 1). The fraction of dimeric ABCC1 appeared to increase as the concentration of the cross-linking reagent DSP increased (compare lanes 1, 3, 5, and 7). Furthermore, the cross-linked 314-kDa protein was reduced to 168 kDa by DTT (lanes 4, 6, and 8), confirming that the 314-kDa protein is likely a cross-linked form of the 168-kDa protein. Thus, human dimeric ABCC1 likely exists in membranes of live cells, and the dimeric proteins detected by
detergent extraction in the above experiments were not due to the detergent and methods of separation used. It is noteworthy that a minor species of cross-linked protein between monomeric and dimeric ABCC1 was also observed at high concentrations of DSP. Currently, it is not known what this minor population is. Apparently, it contains ABCC1 as detected by anti-ABCC1 antibody.

Because a stable cell line with high ectopic expression of ABCC1 was employed in the experiments described above, it is possible that the dimerization of ABCC1 was driven by the high level of the protein in the plasma membranes. To rule out this possibility, we performed another cross-linking experiment using two additional stable cell clones (6-4 and 6-9) (Fig. 5B) with a substantially lower ABCC1 expression level as determined by Western blotting. Fig. 5C shows that dimeric ABCC1 was detected following chemical cross-linking in both of these cell clones. Thus, the formation of dimeric ABCC1 is unlikely dependent on its expression level.

**Human ABCC1 Is a Homodimer**—To further confirm that human ABCC1 exists as a dimer and to determine whether human dimeric ABCC1 is a homodimer, we performed co-immunoprecipitation of human ABCC1 with two different tags. For this purpose, FLAG-tagged human ABCC1 (ABCC1F-FLAG) was transiently transfected into HEK293 cells that did or did not express stable HA-tagged ABCC1 (ABCC1F-HA) (Fig. 1A) as described under “Experimental Procedures.” Western blot analysis of cell lysates showed that both ABCC1F-FLAG and ABCC1F-HA could be coexpressed efficiently in these cells (Fig. 7A, lane 2). We next performed co-immunoprecipitation to determine whether ABCC1F-FLAG interacts with ABCC1F-HA. As shown in Fig. 7A, ABCC1F-FLAG was coprecipitated with ABCC1F-HA by anti-HA antibody (lane 5), whereas ABCC1F-HA alone could not be precipitated by anti-HA antibody (lane 6). On the other hand, the FLAG-tagged carboxyl-terminal core structure
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The Amino-terminal 281 Amino Acids (but Not the Carboxyl-terminal Core Structure) Has Dominant-negative Activity—To further determine the role of ABCC1^{281N} in ABCC1 dimerization, we next performed a functional analysis. It was shown previously that neither ABCC1^{281N} nor the carboxyl-terminal core structure alone is functional for LTC\(_4\) transport (21, 22, 29). We hypothesized that, if ABCC1^{281N} contains the homodimerization domain and can interact with full-length ABCC1 (Fig. 7A), coexpression of ABCC1^{281N} (but not ABCC1^{CORE-FLAG}) with full-length ABCC1 may inhibit the activity of full-length ABCC1 by a dominant-negative effect if the dimeric molecule is a functional unit. To test this hypothesis, we performed an LTC\(_4\) transport assay using membrane vesicles isolated from cells expressing ABCC1^{F-HA} alone, ABCC1^{281N} alone, ABCC1^{F-HA} together with ABCC1^{281N}, ABCC1^{CORE-FLAG} alone, or ABCC1^{F-HA} together with ABCC1^{CORE-FLAG}. Fig. 7 shows that both ABCC1^{281N} and ABCC1^{CORE-FLAG} could be efficiently coexpressed together with ABCC1^{F-HA} as determined using their respective common antibodies. Indeed, Western blot analysis of the isolated membrane vesicles confirmed the expression of these constructs in different combinations and that the expression of full-length ABCC1^{F-HA} was not decreased by the coexpressed domain constructs (Fig. 8, C and F). As shown in Fig. 8 (A and D), both ABCC1^{281N} and ABCC1^{CORE-FLAG} did not have LTC\(_4\) transport activity compared with ABCC1^{F-HA}, which actively accumulated LTC\(_4\) in membrane vesicles, consistent with previous studies (21, 22, 29). However, the LTC\(_4\) transport activity of full-length ABCC1^{F-HA} was decreased by >60% upon coexpression of ABCC1^{281N}, but not ABCC1^{CORE-FLAG}. As shown by Western blotting, the use of similar quantities of proteins was confirmed by Coomassie Blue staining (Fig. 8, B and E). These results are consistent with the coprecipitation experiments (Fig. 7) and confirm that ABCC1^{281N} (but not ABCC1^{CORE}) contains the homodimerization domain. These results also suggest that dimeric ABCC1 may be the functional unit.

**DISCUSSION**

In this study, we used several biochemical approaches to determine the oligomeric status of human ABCC1 and found that ABCC1 likely exists as a homodimer. We also located the (ABCC1^{CORE-FLAG}) did not coprecipitate with ABCC1^{F-HA} (Fig. 7D, lanes 5 and 8) even though both proteins could be precipitated by their respective anti-tag antibodies (lanes 2 and 11) and could be efficiently coexpressed as determined using their common antibody QCRL-1 (Fig. 7E). These results suggest that ABCC1^{281N} (but not ABCC1^{CORE-FLAG}) likely contains the domain that is responsible for ABCC1 homodimerization.

Because the amino-terminal end of ABCC1 contains two sugar chains, we next determined whether glycosylation affects dimerization. ABCC1^{281N} and ABCC1^{F-HA} were cotransfected into HEK293 cells as described above, followed by treatment with tunicamycin to inhibit glycosylation. The cell lysates were then prepared for Western blot and co-immunoprecipitation analyses. We found that the mobility of ABCC1^{281N} following tunicamycin treatment was much faster compared with the major glycosylated bands, but similar to the minor unglycosylated bands shown in Fig. 7A (compare lanes 1 and 2 in Fig. 7B with lanes 2 and 3 in Fig. 7A), suggesting that glycosylation was inhibited. Fig. 7B also shows that unglycosylated ABCC1^{281N} could co precipitate with ABCC1^{F-HA}. Thus, the sugar chains at the amino terminus apparently do not play any role in ABCC1 dimerization. To further verify that ABCC1^{281N} contains the dimerization activity, we coexpressed and co-immunoprecipitated ABCC1^{281N} with two different tags (ABCC1^{281N-FLAG} and ABCC1^{281N-HA}). As shown in Fig. 7C, ABCC1^{281N-HA} coprecipitated with ABCC1^{281N-FLAG} and vice versa. Thus, we conclude that the amino-terminal 281 amino acids of ABCC1 contain a dimerization domain.
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FIGURE 8. Dominant-negative effect of ABCC1281N and ABCC1CORE-FLAG on the function of human ABCC1F-HA. Inside-out membrane vesicles (2 μg) from human HEK293 cells expressing ABCC1F-HA alone (H), ABCC1281N alone (N), ABCC1CORE-FLAG alone (C), ABCC1F-HA together with ABCC1281N (H/N), or ABCC1F-HA together with ABCC1CORE-FLAG (μ/C) were incubated with [3H]LTC4 in the presence of 4 mM ATP or AMP at 25 °C for 2 min as described under “Experimental Procedures.” The results shown in A and D are the means ± S.D. of triplicate determinations in a representative experiment. B and E show similar quantities of proteins used for the LTC4 transport assay. For Western blot (immunoblot (IB)) analysis (C and F), ABCC1F-HA, ABCC1281N, and ABCC1CORE-FLAG were detected by anti-HA antibody, monoclonal antibody MRPr1, and anti-FLAG antibody, respectively.

dimerization domain to the amino-terminal 281 amino acids (ABCC1281N), including MSD0 and L0 (MSD0L0). ABCC1281N can also function as a dominant-negative molecule to inhibit the transport activity of full-length ABCC1 likely by disrupting homodimerization between the two full-length molecules. These observations suggest that MSD0L0 may play an important structural and regulatory role by bringing two ABCC1 molecules together and that dimeric ABCC1 may be the functional unit.

Compared with the half-ABC transporters, the full-length ABC transporters such as ABCB1 were thought to exist and function as monomers. Evidence supporting a functional ABCB1 monomer comes from analysis of two-dimensional crystals of ABCB1 (30) and co-immunoprecipitation of two differentially tagged ABCB1 molecules (31, 32), which suggested that ABCB1 may exist as a monomer. However, dimeric and higher order oligomeric ABCB1 proteins have been shown to exist, and they may be the functional form as determined using radiation inactivation (33, 34), chemical cross-linking and study of the MRK16 epitope (35, 36), and sucrose density gradient sedimentation (37). Clearly, more studies on the functional unit and oligomeric status of ABCB1 are needed.

Unlike ABCB1, the oligomeric status of ABCC1 has not been seriously addressed until now. We have clearly shown here that human ABCC1 exists as a homodimer using multiple approaches, including PFO-PAGE, nondenaturing PAGE, gel filtration chromatography, sucrose density gradient sedimentation, chemical cross-linking, and co-immunoprecipitation. The derivation of dimeric ABCC1 was based on the ratio between the molecular mass measured in the presence of the nondenaturating detergent PFO (ionic) or Triton X-100 (nonionic) and that measured in the presence of SDS, which was assumed to denature ABCC1 into monomers. It is noteworthy that the apparent molecular mass of monomeric ABCC1 in SDS obtained with each method of separation varied from 132 to 289 kDa (Table 1). However, the apparent molecular mass of ABCC1 in PFO or Triton X-100 obtained with all methods of separation was always consistently about twice that in SDS (1.9–2.4-fold). Cross-linked ABCC1 also had an apparent molecular mass that was 1.9 times that of the uncross-linked molecules (Table 1). In a previous imaging analysis of two-dimensional crystals of ABCC1, it appeared that purified ABCC1 crystallized as a homodimer (19). Thus, we conclude that human ABC1 largely exists as a homodimer in cell membranes.

PFO, a mild ionic detergent, was used to extract ABCC1 in most of our approaches. Although PFO has been used successfully in previous studies to extract and preserve the oligomeric status of membrane proteins (see above), the use of high concentrations of this detergent (e.g. 4%) appeared to generate more monomeric ABCC1. Thus, the dimeric ABCC1 protein detected was unlikely due to aggregation caused by PFO. This conclusion is supported by the fact that no putative aggregates bigger than dimeric molecules were observed. It is also supported by the use of the nonionic detergent Triton X-100 in nondenaturing PAGE (Fig. 2) and co-immunoprecipitation (Fig. 6) and in chemical cross-linking experiments in which detergents were not used (Fig. 5). Although the cross-linked molecules could potentially contain other unknown molecules, the facts that the size of the cross-linked molecules is about twice that of monomeric ABCC1 and that differentially tagged ABCC1 proteins co-immunoprecipitate with each other suggest that the cross-linked molecules are homodimers of ABCC1.

Also unlike ABCB1 and most of other full-length ABC transporters such as ABCC7, ABCC1 has an additional MSD (MSD0), the function of which is currently unknown. Although it has been shown that deletion of the entire MSD0 does not affect the trafficking and transport activity of ABCC1 (22), it has also been shown that deletions and mutations of this domain interfere with the processing and transport activity of ABCC1 (20, 21, 38, 39). Most recently, it was found that MSD0 may contain trafficking and/or processing signals (40).

In this study, we found that the first 281 amino acids, including MSD0 and L0 (ABCC1281N), contain the dimerization domain of ABCC1, suggesting that it may play an important structural role by bringing two ABC1 molecules together. Coexpression of MSD0L0 (ABCC1281N), but not the carboxy-terminal core structure (ABCC1CORE-FLAG), inhibited the LTC4 transport activity of full-length ABCC1, indicating that MSD0L0 has dominant-negative activity likely by binding to full-length ABCC1 and preventing it from binding to another full-length molecule to form a functional homodimer. However, it should be noted that the dominant-negative effect of ABCC1281N may also be due to any unknown improper replacement of the amino-terminal domain of the full-length molecule by ABCC1281N. It may also be possible that overexpressed
ABCC1<sup>281N</sup> may compete for drug binding and thus decreased drug uptake in our assays because it has been shown previously that the amino-terminal 281 amino acids contain a drug-binding site (41). However, we think this possibility unlikely because the carboxyl-terminal core structure did not affect drug uptake in our assays, and it also contains drug-binding sites as demonstrated previously (42). Nevertheless, it was found previously using radiation inactivation that the transport of 2,4-dinitrophenyl-S-glutathione in human erythrocytes is possibly through dimeric ABCC1, although there was no evidence for the existence of ABCC1 in these erythrocytes in the study (18). These observations together suggest that human ABCC1 likely functions as a homodimer brought together by MSD0L0. Based on these observations, it is tempting to propose that MSD0L0-mediated dimerization of ABCC1 may be used as a target for developing therapeutics to sensitize ABCC1-induced multidrug resistance in cancer chemotherapy.

Our finding that dimeric ABCC1 is the functional unit and that MSD0L0 is responsible for dimerization is consistent with previous studies demonstrating that human ABCC1 lacking the entire MSD0 region (the amino-terminal 204 amino acids) is still functional in transporting substrates such as LTC<sub>4</sub> (22), whereas the molecule lacking MSD0L0 (the amino-terminal 281 amino acids) is not (21, 22, 29). In this study, MSD0L0 (ABCC1<sup>281N</sup>) contained all of the amino-terminal 281 amino acids. Therefore, it is tempting to speculate that the loop (L0) linking MSD0 and MSD1 (amino acids 204–281) may be involved in ABCC1 dimerization. It should also be noted that the coexpression of MSD0L0 and the core structure of ABCC1 created functional transporters in a previous study (22), suggesting that there is an intramolecular interaction between MSD0L0 and the carboxyl-terminal core structure in addition to the intermolecular interactions between the MSD0L0 domains of two different ABCC1 molecules. We are currently working toward testing these possibilities.

In the ABCC subfamily of human ABC transporters, only ABCC1–3, ABCC6, and ABCC8–10 contain the additional MSD0 region and L0. Although there is no evidence concerning the existence of dimers for these other ABC transporters, the fact that they all contain MSD0L0 suggests that they may also exist as homodimers, as does ABCC1. Interestingly, one of the members of the ABCC subfamily, ABCC7 (also known as the cystic fibrosis transmembrane conductance regulator), which does not have MSD0L0, has been suggested to exist and function as a homodimer in several studies using covalent linkage of two tandem ABCC7 molecules (43), biochemical characterization (44), and imaging analyses with freeze fracture electron microscopy (45) and atomic force microscopy (46). Furthermore, the PDZ domain-containing protein CAP70 (cystic fibrosis transmembrane conductance regulator-associated protein 70) was found to facilitate the homodimerization of ABCC7 (47).

Formation of dimers or higher order oligomers may help create another level of regulation of the function of ABC transporters (17). It is unknown whether there is any mechanism that regulates the formation of dimers or oligomers of ABC transporters. It is also unknown whether the expression level plays any role in regulating the formation of dimers or oligomers. It is possible that a high expression level drives the dimerization of these proteins. However, we found that the expression level of ABCC1 did not appear to affect the formation of ABCC1 dimers. Additional studies are clearly needed to address these issues and to determine whether other full-length ABC transporters exist and function as homodimers.

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