Characterization of Oligomeric Human Half-ABC Transporter
ATP-binding Cassette G2*

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Human ATP-binding cassette G2 (ABCG2, also known as mitoxantrone resistance protein, breast-cancer-resistance protein, ABC placenta) is a member of the superfamily of ATP-binding cassette (ABC) transporters that have a wide variety of substrates. Overexpression of human ABCG2 in model cancer cell lines causes multidrug resistance by actively effluxing anticancer drugs. Unlike most of the other ABC transporters which usually have two nucleotide-binding domains and two transmembrane domains, ABCG2 consists of only one nucleotide-binding domain followed by one transmembrane domain. Thus, ABCG2 has been thought to be a half-transporter that may function as a homodimer. In this study, we characterized the oligomeric feature of human ABCG2 using non-denaturing detergent perfluoro-octanoic acid and Triton X-100 in combination with gel filtration, sucrose density gradient sedimentation, and gel electrophoresis. Unexpectedly, we found that human ABCG2 exists mainly as a tetramer, with a possibility of a higher form of oligomerization. Monomeric and dimeric ABCG2 did not appear to be the major form of the protein. Further immunoprecipitation analysis showed that the oligomeric ABCG2 did not contain any other proteins. Taken together, we conclude that human ABCG2 likely exists and functions as a homotetramer.

Multidrug resistance (MDR)† is a major problem in successful cancer chemotherapy. One of the known causes of MDR is overexpression of drug efflux pumps on cancer cell surfaces that actively extrude anticancer drugs out of cells and, thus, eliminate effective intracellular accumulation of these drugs. Human ABCG2 is one of these drug-efflux pumps that can actively transport a wide variety of anticancer drugs, including anthracyclines (1). Human ABCG2 is a member of the ATP-binding cassette (ABC) membrane transporter superfamily that also includes other drug-efflux pumps, such as P-glycoprotein (Pgp) and multidrug resistance protein 1 (MRP1). There are seven subfamilies of human ABC transporters, and ABCG2 belongs to the ABCG subfamily (see the web-sites www.gene.ucl.ac.uk/nomenclature/genefamily/abc.html and nutrigen. 4t.com/humanabc.htm for a complete list).

Human ABCG2, also known as BCRP, MXR, and ABCP (referred to as ABCG2 in the remaining text), was initially identified and cloned by three independent groups (1–3). Overexpression of ABCG2 in model cancer cell lines has been shown to cause resistance to anthracycline daunorubicine and anthracyclenes mitoxantrone (1), similar to that of the selected drug-resistant MCF7/AdrVp3000 cells, which overexpress ABCG2. ABCG2 shares a spectrum of drug substrates with two other well-known MDR-causing ABC transporters, Pgp and MRP1 (4–6). Like other members of the human ABCG subfamily, ABCG2 is a half-transporter with a unique arrangement of its nucleotide-binding domain (NBD) and transmembrane domain (TMD). However, unlike most ABC transporters in other ABC subfamilies, which usually have a common domain organization of TMD1-NBD1-TMD2-NBD2, ABCG2 has an organization of NBD-TMD, with the NBD located at the amino terminus.

ABCG2 has been thought to function as a homodimer (1, 2, 5) because (i) its size is about half that of many other ABC transporters; (ii) its homologous proteins in Drosophila (white, brown, and scarlet eye pigment transporters) have been shown to work as heterodimers (7, 8); and (iii) its human homologous proteins ABCG5 and ABCG8 were reported to form heterodimers in the endoplasmic reticulum prior to being transported to the apical membranes, which is important for their function of promoting the excretion of neutral sterols (9). The hypothesis that ABCG2 functions as a homodimer was supported by a study which showed that two differentially tagged ABCG2s co-precipitated with each other and that disulfide bonds existed between the two ABCG2 subunits (10). Human ABCG2 has been functionally expressed in both insect and bacteria cells, suggesting that no other mammalian partner may be needed for ABCG2 function (11–13).

However, it has been found by chemical cross-linking that higher forms of oligomeric ABCG2 may exist in drug-resistant cells that express ABCG2 (14). Although it remains controversial, some studies have suggested that both human MRP1 and Pgp may exist and function as a homodimer (15–19). Thus, it is possible that human ABCG2 may also exist and function as an...
oligomer such as a tetramer. To test this hypothesis, perfluoro-octanoic acid (PFO) and Triton X-100 were used to extract ABCG2 from MCF-7/AdrVp3000 cells that overexpress ABCG2; the oligomeric status of ABCG2 was determined using PFO-PAGE, sucrose density gradient sedimentation, gel filtration chromatography, and immunoprecipitation. We found that the monomeric and homodimeric ABCG2s did not exist as major forms and that most human ABCG2s exist as homotetramers in plasma membranes.

EXPERIMENTAL PROCEDURES

Materials—PFO was purchased from Oakwood Products. Disuccinimidyl suberate (DSP) was obtained from Pierce Biotechnology. Monoclonal antibody BXP-21 against ABCG2, anti-HA antibody, and protein G-Sepharose 4B from BioRad were from ICN, Covance, and Santa Cruz Biotechnology, respectively. Adriamycin, verapamil, β-galactosidase marker, and Triton X-100 were obtained from Sigma. The enhanced chemiluminescence system for Western blot analysis, Superose 6 HR column, thyroglobulin, catalase, bovine serum albumin (BSA), ovalbumin, and ribonuclease A were from Amersham Biosciences. Polyvinylidene difluoride membranes, concentrated protein assay dye reagents, and precast polyacrylamide gradient gels were from BioRad. Laminin and [35S]methionine were from Amersham Biosciences. Polyvinylidene difluoride membranes, and ABCG2 was detected using the monoclonal antibody BXP-21. [35S]methionine from ICN, PerkinElmer Life Sciences, respectively. Cell culture media and reagents were obtained from either Invitrogen or Cambrex Bioscience Walkersville. All other reagents of molecular biology grade were purchased from Sigma or Fisher.

Cell Culture and Plasma Membrane Preparation—MDR1 cell lines MCF-7/AdrVp3000 and S1–M1–80 were grown at 37 °C with 5% CO2 in IMEM and RPMI 1640 medium, respectively, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin. Crude and BHK cells, as described previously (22, 23). The membrane pellet was resuspended in STBS (250 mM sucrose, 150 mM NaCl, and 10 mM Tris, pH 7.5) and stored at −80 °C. The protein concentration of the membrane pellets was determined using BioRad protein assay kit.

PFO-PAGE Analysis—Extraction of ABCG2 from membranes with PFO and PFO-PAGE were performed as described by Ramjeesingh et al. (24) with minor modification. Briefly, 2–4 g of plasma membranes in STBS 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, 0.5–8% PFO) followed by addition of dithiothreitol (DTT) to a final concentration of 5 mM and incubated at room temperature for 30 min. After centrifugation at 11,000 × g for 10 min, the supernatants were loaded onto the freshly prepared 7.5% Tris/glycine polyacrylamide gel without SDS. Electrophoresis was performed at 100 V at 4 °C using the running buffer containing 25 mM Tris, pH 8.5, 192 mM glycine, and 0.1% PFO. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes, and ABCG2 was detected using the monoclonal antibody BXP-21 by enhanced chemiluminescence, as described previously (22).

Non-denaturing Gel Electrophoresis—10 g of plasma membranes in STBS were mixed with an equal volume of 2× Triton X-100 extraction sample buffer (100 mM Tris, pH 8.0, 20% glycerol, 0.005% bromphenol blue, 2% Triton X-100, and 100 mM DTT) followed by incubation at room temperature for 30 min. After centrifugation at 11,000 × g for 10 min, the supernatants were loaded onto the precast 4–15% gradient Tris/ glycine polyacrylamide gel. Electrophoresis was performed at 140 V at room temperature with running buffer (250 mM glycine and 25 mM Tris, pH 8.3) followed by transfer to polyvinylidene difluoride membrane for Western blot analysis, as described previously (22).

Gel Filtration Chromatography—Gel filtration chromatography was performed on an AKTA purifier system (Amersham Biosciences) with a Superose 6 HR column. 30 μg of plasma membranes were solubilized by 0.5% PFO, 4% PFO, 1% Triton X-100, or 1% SDS in the buffer containing 50 mM Tris-HCl, pH 8.0, and 100 mM DTT for 30 min at room temperature. After clearance of insoluble materials by centrifugation at 11,000 × g for 10 min, the supernatants were injected into the column equilibrated with the running buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM DTT, and 0.1% PFO, Triton X-100, or SDS). The elution was collected at 1 ml or 0.5 ml per fraction and precipitated with 10% trichloroacetic acid. The retention time of ABCG2 and HA-tagged CFTR was determined by SDS-PAGE and Western blotting of the collected fractions. Protein markers, including thyroglobulin (669 kDa), laminin (400 kDa and 200 kDa in the presence of DTT), catalase (232 kDa), β-galactosidase (116 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and ribonuclease A (13.7 kDa) (Amersham Biosciences), were separated by AKTA purifier under the same conditions and detected by the UV detector of the AKTA purifier.

Sucrose Density Gradient Sedimentation—ABCG2 was first extracted from 30 μg of plasma membranes as described above. After clearance by centrifugation, the extracts were layered atop a continuous 20–35% (w/v) sucrose density gradient in a buffer containing 50 mM Tris-HCl, pH 7.4, 1 mM DTT, and 0.1% PFO, 0.1% Triton X-100, or 0.1% SDS. The sedimentation was performed with a Beckman SW41 rotor at 100,000 × g for 18 h at 4 °C. Fractions were then collected at 0.5 ml each, and proteins were precipitated with 10% trichloroacetic acid followed by SDS-PAGE and Western blot analysis to determine the relative position of ABCG2. Protein markers including thyroglobulin (669 kDa), catalase (232 kDa), and bovine serum albumin (67 kDa) were separated and fractionated under the same conditions and detected by SDS-PAGE and Coomassie Blue staining.

Chemical Cross-linking—Chemical cross-linking using DSS was performed as described previously (14). Confluent cells were washed twice with the KCl/Hepes buffer (90 mM KCl, 50 mM Hepes, pH 7.5) and incubated with 2 mM DSS in the KCl/Hepes buffer with shaking on a nutator for 45 min at 22 °C. Glycine was then added to a final concentration of 2 mM before cells were collected for membrane preparation, followed by separation on a 4–15% continuous gradient precast gel and Western blot analysis.

Metabolic Labeling and Immunoprecipitation—About 3 × 106 MCF-7/AdrVp3000 cells were seeded in a 60-mm dish and cultured for 3 days. The medium was then removed, and the cells were washed twice with PBS and once with DMEM lacking methionine, followed by incubation in the same medium supplemented with 80 μCi [35S]methionine for 8 h. The labeled cells were then washed 3 × with PBS and harvested for lystate preparation using the lysis buffer containing 150 mM NaCl, 50 mM Tris, pH 8.0, 2 mM phenylmethylsulfonyl fluoride, and 0.5% PFO or 1% Triton X-100. The cell lystate was cleared of insoluble materials by centrifugation and used for immunoprecipitation as described previously (25). Briefly, 100 μg of cell lysates were mixed with 10 μl of 50% protein G-Sepharose 4B slurry; bovine serum albumin at the final concentration of 2% was included. The mixture was incubated at 4 °C for 1 h and centrifuged to remove Sepharose beads together with nonspecifically bound proteins. 5 μg of anti-ABCG2 monoclonal antibody BXP-21 or normal mouse IgG were added to the supernatant and incubated at 4 °C for 3 h before mixing with 30 μl of 50% protein G-Sepharose 4B slurry. The mixture was further incubated for 3 h or overnight at 4 °C with agitation. The immunoprecipitate was collected by centrifugation and washed six times with the PFO or Triton X-100 containing lysis buffer. The final pellet was solubilized in 10 μl of sample buffer for SDS-PAGE and autoradiography.

RESULTS

Formation of Intermolecular Disulfide Bonds between Human ABCG2 Subunits—In previous studies, it was shown that the human ABCG2 exists as a homodimer with intermolecular disulfide bonds. However, we consistently found that very little

Fig. 1. Dimeric ABCG2 due to formation of intermolecular disulfide bonds. 40 μg of freshly prepared cell lystate (lanes 1 and 2) and 10 μg of plasma membranes (lanes 3 and 4) from MCF-7/AdrVp3000 cells were solubilized in SDS sample buffer with (lanes 1 and 3) or without (lanes 2 and 4) 100 mM DTT followed by separation on SDS-PAGE and Western blot analysis using monoclonal antibody BXP-21. Membr.
or no dimeric human ABCG2 from cell lysate could be detected on non-reducing SDS-PAGE whereas such dimeric ABCG2 was found only with the isolated membranes (Fig. 1). We also found that at best, only about 50% of ABCG2 in isolated membranes have the intermolecular disulfide bonds for the formation of dimers. This observation suggests that the disulfide bond between the ABCG2 subunits was likely formed by oxidation during cell lysis and membrane preparation; it thus raises a question whether human ABCG2 really forms a homodimer in live cells. Furthermore, oligomeric ABCG2 larger than a homodimer was observed after chemical cross-linking in a previous study (14). Thus, it is necessary to investigate whether human ABCG2 is a dimer and whether other oligomeric ABCG2s exist. To prevent the potential problem introduced by the formation of an intermolecular disulfide bond, DTT was employed in all experiments described in the following studies.

**PFO Extraction and PFO-PAGE Analysis of Human ABCG2**—To investigate the oligomeric status of human ABCG2, we first extracted the method of PFO-PAGE. PFO-PAGE has been used successfully for studying the oligomeric status of both water soluble proteins (26, 27) and membrane proteins (24, 28, 29) by using a mild ionic detergent, PFO. PFO at appropriate concentrations does not break the non-covalent interactions between protein subunits of an oligomer and, therefore, permits the extraction and determination of oligomeric states of membrane proteins by polyacrylamide gel electrophoresis or PFO-PAGE.

We first tested the optimal PFO concentration to extract human ABCG2 from plasma membranes of MCF7/AdrVp3000 cells using SDS-PAGE and Western blot. As shown in Fig. 2A, PFO at concentrations of 0.5% and above successfully extracted most ABCG2, and the low speed centrifugation (11,000 × g) separated the extracted ABCG2 from the insoluble pellet, whereas 0.25% PFO did not extract ABCG2 from membranes (lanes 1–12). On the other hand, the majority of ABCG2 remained with membrane pellet after low speed centrifugation if untreated by PFO (Fig. 2A, lanes 13–14). Therefore, we used 0.5 and 4% PFO to extract ABCG2 for further oligomeric analysis. Fig. 2B shows the results of PFO-PAGE of ABCG2 extracted by 0.5% and 4% PFO followed by Western blotting analysis. Two major diffused bands of ABCG2 with apparent molecular masses of ~285 kDa and ~799 kDa were detected when extracted by 0.5% PFO (Fig. 2B, lane 1), whereas only the 285-kDa band was observed when extracted by 4% PFO (Fig. 2B, lane 2). Assuming that the monomeric ABCG2 has an apparent molecular mass of ~72 kDa as estimated using SDS-PAGE (30) (see also discussions below), we calculated that the 285-kDa band is likely a tetramer, whereas the 799-kDa band is likely a higher order of oligomerization consisting of ~11–12 subunits (see Table I). Interestingly, we did not detect any

| Methods of separation | Mr | No. of subunits | Oligomeric state |
|-----------------------|----|----------------|-----------------|
| PFO-PAGE              |    |                |                 |
| 0.5% PFO             | 799 | 11.1           | Dodecamer       |
| 4% PFO               | 285 | 4              | Tetramer        |
| Gel filtration        |    |                |                 |
| 0.5% PFO             | 265 | 3.7            | Tetramer        |
| 4% PFO               | 265 | 3.7            | Tetramer        |
| 1% SDS                | 60  | 0.8            | Tonomer         |
| 1% TX-100             | 901 | 12.5           | Dodecamer       |
| 4% PFO (CFTR)         | 355 | 2.1*           | Dimer           |
| 1% SDS (CFTR)         | 219 | 1.3*           | Monomer         |
| Gradient sedimentation|    |                |                 |
| 0.5% PFO             | 203 | 2.8 (4.0)*     | Tetramer        |
| 4% PFO               | 203 | 2.8 (4.0)      | Tetramer        |
| 1% SDS                | 51  | 0.7 (1.0)      | Monomer         |
| 1% TX-100             | 518 | 7.2 (10.3)     | Dodecamer       |
| Non-denaturing PAGE   |    |                |                 |
| 1% TX-100             | 806 | 11.2           | Dodecamer       |
| SDS-PAGE              |    |                |                 |
| −DSS                  | 73  | 1.0            | Monomer         |
| +DSS                  | 73  | 1.0            | Monomer         |
| −DSS                  | 143 | 2.0            | Dimer           |
| +DSS                  | 224 | 3.1            | Trimer          |
| +DSS                  | 287 | 4.0            | Tetramer        |

* The size of ABCG2 used for calculation is 72 kDa, based upon previous observations from SDS-PAGE (30), whereas 170 kDa was used for the calculation of CFTR (31). The theoretical molecular mass was not used because of the sugar chains of the protein, for which the mass contribution is unknown.

* The numbers in parentheses are normalized against that of the size of ABCG2 determined in the presence of SDS, which is presumably monomeric.

ABC2G2 with the size of a dimer on PFO-PAGE. Thus, the minimum unit of the oligomeric human ABCG2 is likely a tetramer and not a dimer as previously thought. A higher degree of oligomerization of ABCG2 may also exist. Similar results were also observed with ABCG2 from another cell line S1-M1–80 (data not shown), suggesting that the oligomeric ABCG2 observed was not due to the use of a specific cell type.

**Gel Filtration Chromatography Analysis of Human ABCG2 in PFO**—The oligomeric status of human ABCG2 was further analyzed by gel filtration chromatography using a Superose 6 HR column following extraction using 0.5 and 4% PFO. As shown in Fig. 3, A and B, human ABCG2 extracted with both 0.5 and 4% PFO was detected between fractions with retention times of 11 ml and 17 ml, respectively, with a peak in the fraction of 14 ml. The peak fraction with retention time of 14 ml has an estimated molecular mass of 265 kDa (Fig. 3D), which
corresponds to a tetramer (Table I). This observation is consistent with that shown by PFO-PAGE above (see Fig. 2B and Table I). As a control, ABCG2 extracted by SDS has an estimated molecular mass of 60 kDa (Fig. 3, E–G), which corresponds to the size of a monomeric ABCG2 (Table I). Interestingly, the higher form of oligomerization in the presence of 0.5% PFO detected on PFO-PAGE was not detected here using gel filtration chromatography. The reason for this discrepancy is currently unknown, but it may possibly be due to the use of different methods of separation (see “Discussion” below).

To further confirm that the estimated size of human ABCG2 in PFO corresponds to a tetramer, we tested the elution profile of human CFTR, another ABC transporter, which has been shown to exist mainly as a homodimer in isolated mammalian plasma membranes (24, 31). As shown in Fig. 3D, the majority of human CFTR in 0.5% PFO was eluted with a retention time of 13.5 ml and an estimated size of 355 kDa, corresponding to a homodimer (Table I). The behavior of CFTR in 1% SDS was also determined as described above for ABCG2. As shown in Fig. 3G, CFTR in SDS was eluted with a retention time of 10 ml; CFTR in SDS has a calculated size of 219 kDa, corresponding to a monomer (Table I). Based on the observations of the CFTR elution profile, we conclude that human ABCG2 separated by gel filtration in PFO is likely a tetramer.

**Sucrose Density Gradient Sedimentation Analysis of Human ABCG2 in PFO**—We next employed sucrose-gradient sedimentation to determine the oligomeric status of human ABCG2. ABCG2 was first extracted with 0.5 and 4% PFO from membranes of MCF-7/AdrVp3000 cells and then subjected to sucrose-gradient sedimentation followed by fractionation, SDS-PAGE, and Western blot for detection. As shown in Fig. 4, A and B, ABCG2 was detected between fractions 13 and 20 with a peak in fraction 17, which has an estimated molecular mass of 203 kDa (Fig. 4D). As a control, ABCG2 extracted with 1% SDS was detected between fractions 19 and 22 with a peak in fraction 21 (Fig. 4C), which has an estimated molecular mass of 219 kDa (Fig. 3G).
The size of the 203-kDa fraction in the presence of PFO is smaller than the calculated size of a tetrameric human ABCG2 (see Table I). This is likely because of underestimation by sucrose-gradient sedimentation; the size of the monomeric ABCG2 determined in the presence of SDS is 30% smaller than the calculated one (see Table I and "Discussion" below). Correction using the value of the monomeric ABCG2 showed that the 203-kDa fraction is likely a tetramer (Table I). It is also noteworthy that the higher form of oligomeric ABCG2 detected using PFO-PAGE was not detected by the use of sucrose-gradient sedimentation. This may be because of the possibility that the higher form of oligomeric ABCG2 is not stable in PFO and is dissociated during the long duration (18 h) of centrifugation (see "Discussion" below).

**Analysis of ABCG2 in Triton X-100**—In all of the above experiments, the mild ionic detergent PFO was used to extract ABCG2 from membranes. To determine that the formation of oligomeric ABCG2 was not due to the use of PFO, the nonionic detergent Triton X-100 was used to extract ABCG2 for gel filtration and sucrose-gradient sedimentation analysis. As shown in Fig. 5A, human ABCG2 in Triton X-100 was eluted in gel filtration between fractions with retention times of 9.5 ml and 14 ml. The peak fraction has a retention time of 12 ml and an estimated molecular mass of 901 kDa (Fig. 5C). It is likely a dodecamer (Table I), which is consistent with the observation produced by PFO-PAGE (see Fig. 2B and Table I). The finding that essentially all ABCG2s are dodecameric and no tetrameric ABCG2 exists in Triton X-100 suggests that the dodecameric ABCG2 may be more stable in Triton X-100 than in PFO. Consistent with this conclusion, we also found that ABCG2 in Triton X-100 has a mobility of 806 kDa separated by non-denaturing gel electrophoresis (Fig. 5E and Table I), suggesting that human ABCG2 in Triton X-100 is likely a high order of complex with 11–12 subunits.
In sucrose-gradient sedimentation, ABCG2 extracted by Triton X-100 was detected between fractions 8 and 16, with a major peak in fraction 14 and a minor peak in fraction 11. These two peaks were estimated to have molecular masses of 518 kDa and 218 kDa, respectively (Fig. 5D). Although the major peak of 218 kDa may be the tetrameric ABCG2 (Table I), which is consistent with that found using PFO (Fig. 4), the minor peak of 518 kDa is likely a higher order of oligomeric ABCG2, possibly a dodecamer (Table I). The detection of the tetrameric ABCG2 in Triton X-100 by sucrose-gradient sedimentation but not by gel filtration chromatography suggests that the long duration of centrifugation may cause dissociation of the higher order of oligomeric ABCG2 (see “Discussion”).

Chemical Cross-linking of Oligomeric ABCG2 in Live Cells—The above studies showed that human ABCG2 likely exists as a tetramer with the possibility of existence as a dodecamer. However, all of the above studies used isolated membranes, and a mild detergent had to be used to preserve the non-covalent protein-protein interactions. To determine further whether the oligomeric ABCG2 exists in live cells without the use of detergent, chemical cross-linking was employed as described previously (14). DSS is a lipophilic and bifunctional cross-linking reagent with an arm length of 11.4 Å. If ABCG2 in live cells exists as an oligomer, it may be cross-linked by DSS and can then be analyzed and detected by SDS-PAGE and Western blotting, as described under “Experimental Procedures.”

As shown in Fig. 6A, three bands of ABCG2 with molecular masses greater than that of the monomeric ABCG2 were clearly detected following cross-linking by DSS, as compared with the control without DSS. The estimated size of these ABCG2 bands are 287, 224, 143, and 73 kDa (Fig. 6C), which corresponds to the sizes of tetrameric, trimeric, dimeric, and monomeric ABCG2s, respectively (Table I). We also noted that the cross-linking efficiency decreases with the increase in complexity of the oligomer (Fig. 6B) and, thus, the more complex forms of oligomeric ABCG2 may not be cross-linked and detectable (see “Discussion”).
ABCG2 is a homo- or hetero-oligomer, we applied an immunoprecipitation assay following metabolic labeling of MCF-7/AdrVp3000 cells with [35S]methionine. Because both Triton X-100 and PFO have been shown to preserve the oligomeric states of human ABCG2 molecules, these two detergents were used to lyse cells. As shown in Fig. 7, no other proteins except human ABCG2 were precipitated specifically by the monoclonal antibody BXP-21 in the presence of Triton X-100 or PFO.

Thus, it is likely that the oligomeric ABCG2 consists of only ABCG2 subunits.

**DISCUSSION**

Compared with most of other ABC transporters such as MRP1 and Pgp, ABCG2 is about half the size and is known as a half-transporter. Based on its half-size, ABCG2 is thought to work as a homodimer (5, 10). However, in this study we demonstrated that the major oligomeric unit of ABCG2 that exists in live cells is a homo-tetramer with a possible higher order of oligomerization consisting of 12 subunits. We found no evidence that any homodimeric or monomeric ABCG2 exists as a major form in plasma membranes.

Although the tetrameric ABCG2 in the presence of PFO could be detected by all methods employed, the dodecameric ABCG2 in PFO could be detected only by PFO-PAGE. On the other hand, the dodecameric ABCG2 in Triton X-100 was easily detectable. The results shown in Fig. 2B suggest that the dodecameric ABCG2 in PFO represents ~55% of total oligomeric ABCG2 detected, whereas the data shown in Fig. 5, A and E suggest that essentially all ABCG2s in Triton X-100 are dodecameric. Interestingly, the dodecameric ABCG2 can hardly be detected when sucrose-gradient sedimentation was used. Because the duration of centrifugation is very long (18 h), the dodecameric ABCG2 may be dissociated. This argument is supported by detection of a small amount of dodecameric ABCG2 in sucrose-gradient sedimentation in the presence of Triton X-100 (Fig. 5B). Interestingly, the dodecameric ABCG2 was not detected by chemical cross-linking in live cells. We have tried other cross-linking reagents with different arm length and function groups (e.g., 3,6,9,12,15-pentaoxaheptadecane-1, 17-diylibismethanethiosulfonate, Bis [sulfosuccinimidyl] suberate, N-sulfosuccinimidyl-6-[4'-azido-2'-nitrophenylamino] hexanoate, and sulfo-N'-succinimidyl-6-[4'-azido-2'-nitrophenylamino] hexanoate) and were unable to detect the cross-linked dodecamers. These observations are likely because of the low efficiency of cross-linking of ABCG2. Fig. 6B shows that only about 35% of ABCG2 are cross-linked as dimers and the cross-linking efficiency of higher forms decreases with the addition of each subunit. Considering the fact that the cross-linking efficiency decreased about 33% on average with the increase in size of one additional subunit (see Fig. 6B), we estimated the efficiency of cross-linking 12 subunits together to be 0.0015%. With this low efficiency of cross-linking, the dodecameric ABCG2 is highly unlikely to be detected by Western blotting. It is also possible that the dodecameric ABCG2 is a nonspecific aggregation of the tetrameric ABCG2, either because of membrane isolation or because of the use of detergents. However, we think that this possibility is unlikely. If the use of detergents and membrane isolation cause nonspecific aggregation of ABCG2, we should have detected the dodecameric ABCG2 using all the methods employed.

Three detergents, SDS, PFO, and Triton X-100, were used in this study to extract ABCG2 from cell membranes. Except SDS which denatures the protein, both PFO and Triton X-100 seem to fully preserve the tetramer of ABCG2. The detection of tetrameric ABCG2 was not affected by the use of these two detergents, suggesting that the formation of tetrameric ABCG2 is not due to the use of various detergents. However, it is noteworthy that although only tetrameric ABCG2 was detected in the presence of 4% PFO, the additional dodecameric ABCG2 was also detected in the presence of 0.5% PFO (Fig. 2B). This observation suggests that the use of higher concentration of PFO may disrupt the formation of the dodecameric ABCG2.
Hence, it is possible that the protein-protein interactions between the tetrameric units to generate the dodecamers (Fig. 8, interface B) are weaker than that between the monomeric units to generate tetramers (Fig. 8, interfaces A and C), and that the tetrameric ABCG2 is likely more stable than the dodecameric ABCG2. This conclusion is also supported by the observation that the dodecameric ABCG2 was not observed and was possibly dissociated into tetramers after the extended centrifugation in the sucrose-gradient studies (Fig. 4) and in gel filtration analysis (Fig. 3). It is noteworthy that all ABCG2s in Triton X-100 were detected as dodecamers in the gel filtration chromatography (Fig. 5A) and in non-denaturing gel electrophoresis (Fig. 5E), and a small fraction of dodecameric ABCG2 was detected in the sucrose-gradient sedimentation (Fig. 5B). Thus, it is likely that the dodecameric ABCG2 is more stable in the presence of Triton X-100 than that in the presence of PFO.

Four different conventional methods of separation were used in this study to analyze the oligomeric status of human ABCG2. Although each method has its own advantages and disadvantages, they complement each other. The use of sucrose-gradient sedimentation may underestimate the size of the oligomeric ABCG2. As shown in Table I, the major peak fractions containing ABCG2 in both PFO and Triton X-100 separated by sucrose-gradient sedimentation have an estimated molecular mass of an oligomer of three subunits of ABCG2, and the minor peak in the presence of Triton X-100 was estimated to have seven subunits. It is possible that the two forms of oligomer may, in fact, be a tetramer and a dodecamer, respectively, considering that the monomeric ABCG2 extracted by SDS was estimated to be only 51 kDa, about 30% less than the known value of 72 kDa (see Table I). This observation also suggests that the ABCG2 complex is tightly packed so that it sediments faster than normal globular proteins.

Although the dodecameric ABCG2 was detected by some methods, the tetrameric ABCG2 seems to be the major form that can be detected by all methods employed in this study. On the other hand, the monomeric and dimeric ABCG2 did not seem to be a major form detected by any of the methods used under non-denaturing conditions. This discovery raises the speculation that the tetrameric or dodecameric ABCG2 may be the functional form of ABCG2. It is also possible that one form may function as a transporter, whereas the other may function by serving as a regulator for the level of the functional ABCG2.

Fig. 7. Immunoprecipitation of ABCG2 in PFO and Triton X-100. 35S-labeled MCF-7/AdrVp3000 cells were treated with PFO (lanes 1 and 2) or Triton X-100 (lanes 3 and 4) containing lysis buffer. ABCG2 (indicated by arrowsheads) was then precipitated from the cell lysate using monoclonal antibody BXP-21 (lanes 1 and 3) or normal mouse IgG (lanes 2 and 4) followed by separation by SDS-PAGE for autoradiography as described under “Experimental Procedures.” N-IgG, normal mouse IgG.

Fig. 8. Schematic organization of dodecameric ABCG2. Three different types of interfaces exist between ABCG2 subunits (A, B, C) in the dodecameric ABCG2 complex. The minimum stable ABCG2 complex is a homotetramer (boxed). The sulphydryl groups (-SH) between the ABCG2 subunits are active and may form disulfide bonds upon oxidation.

Formation of one form of oligomer may decrease the level of the other and, thus, decrease the membrane level of active ABCG2 transporters. However, it is also possible that the dimeric ABCG2 is a functional drug transporter as previously thought, and that the tetrameric and dodecameric ABCG2s function only as a regulator for the level of functional dimeric ABCG2 transporters. We are currently investigating these possibilities. It is not presently known whether there is any intermolecular disulfide bond between ABCG2 subunits in live cells. However, it seems that the intermolecular disulfide bond that formed during membrane isolation exists only between the two ABCG2 subunits. There are no intermolecular disulfide bonds that link a tetramer of ABCG2 together. Thus, it is possible that the interface (Fig. 8, interface A) between the ABCG2 subunits that has a disulfide bond is different from that between the two dimeric units (Fig. 8, interface C). Because the tetrameric ABCG2 is more stable than the dodecameric ABCG2 as discussed above, it is also possible that the interactions between the tetrameric units (Fig. 8, interface B) are different from those between the dimeric units (Fig. 8, interfaces A and C). Although the disulfide bonds may not be formed physiologically, the formation of these bonds when oxidized indicates that the interface between the two ABCG2 subunits contains active cysteine residues. Further studies on the cysteine residues may help reveal the protein domains that are involved in forming tetrameric and dodecameric ABCG2. These domains may be used as targets for designing probes that can be used to inhibit ABCG2 oligomerization and, thus, possibly for better efficacy in cancer chemotherapy.

If, as demonstrated in this study, human ABCG2 exists as a homotetramer, it raises the possibility that the other full-size ABC drug transporters such as human MRP1 and Pgp may also exist as homodimers. Indeed, Szollosy et al. (15) showed (using radiation inactivation assay) that MRP1 in human erythrocytes may function as a dimer. This observation is consistent with the image analysis of two-dimensional crystals of purified MRP1 by electron microscopy which showed that MRP1 crystallized as a homodimer (16). Dimeric Pgp has been shown to exist and may be the functional form using radiation inactivation (17, 18), chemical cross-linking, and analysis of MRK-16 epitope (32, 33). Dimers and higher orders of oligomeric Pgp have also been shown to exist after CHAPS extraction and sucrose-gradient sedimentation (19). However, the existence of dimeric Pgp has been argued against by other studies. Rosenberg et al. (34) found that, unlike MRP1, the two-dimensional crystals of Pgp are monomeric (34). Differentially
tagged Pgp molecules were found not to co-precipitate with each other if co-expressed, suggesting that they likely function as monomers (35, 36). Clearly, further studies are needed to determine whether the dimeric Pgp exists and whether it has any functional role.

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REFERENCES

1. Doyle, L. A., Yang, W., Abruzzo, L. V., Krogmann, T., Gao, Y., Rishi, A. K., and Ross, D. D. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15665–15670
2. Miyake, K., Mickley, L., Litman, T., Zhan, Z., Robey, R., Cristensen, B., Branghi, M., Greenberger, L., Dean, M., Fojo, T., and Bates, S. E. (2001) J. Biol. Chem. 276, 16076–16082
3. Allikmets, R., Schriml, L. M., Hutchinson, A., Romano-Spica, V., and Dean, M. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 959–964
4. Ozvegy, C., Litman, T., Szakacs, G., Nagy, Z., Bates, S., Varadi, A., and Sarkadi, B. (2002) Biochim. Biophys. Acta 1565, 6–16
5. Boscohoziník, D., Debanne, M. T., Stafford, A. R., Jung, C. Y., Gupta, R. S., and Epand, R. M. (1999) Biochim. Biophys. Acta 1327, 229–238
6. Jette, L., Potier, M., and Beliveau, R. (1997) Biochemistry 36, 13929–13937
7. Peruchynsky, M. S., and Ling, V. (1994) Biochemistry 33, 4163–4174
8. Boscoboinik, D., Debanne, M. T., Stafford, A. R., Jung, C. Y., Gupta, R. S., and Epand, R. M. (1999) Biochim. Biophys. Acta 1327, 229–238
9. Jette, L., Potier, M., and Beliveau, R. (1997) Biochemistry 36, 13929–13937
10. Peruchynsky, M. S., and Ling, V. (1994) Biochemistry 33, 4163–4174
11. Boscoboinik, D., Debanne, M. T., Stafford, A. R., Jung, C. Y., Gupta, R. S., and Epand, R. M. (1999) Biochim. Biophys. Acta 1327, 229–238
12. Miksic, E., Tsuruo, T., Ling, V., and Remmer, M. (2002) Biochim. Biophys. Acta 1565, 6–16
13. Boscohoziník, D., Debanne, M. T., Stafford, A. R., Jung, C. Y., Gupta, R. S., and Epand, R. M. (1999) Biochim. Biophys. Acta 1327, 229–238
14. Jette, L., Potier, M., and Beliveau, R. (1997) Biochemistry 36, 13929–13937
15. Peruchynsky, M. S., and Ling, V. (1994) Biochemistry 33, 4163–4174
16. Boscoboinik, D., Debanne, M. T., Stafford, A. R., Jung, C. Y., Gupta, R. S., and Epand, R. M. (1999) Biochim. Biophys. Acta 1327, 229–238
17. Jette, L., Potier, M., and Beliveau, R. (1997) Biochemistry 36, 13929–13937
18. Peruchynsky, M. S., and Ling, V. (1994) Biochemistry 33, 4163–4174
19. Boscoboinik, D., Debanne, M. T., Stafford, A. R., Jung, C. Y., Gupta, R. S., and Epand, R. M. (1999) Biochim. Biophys. Acta 1327, 229–238
20. Jette, L., Potier, M., and Beliveau, R. (1997) Biochemistry 36, 13929–13937
21. Peruchynsky, M. S., and Ling, V. (1994) Biochemistry 33, 4163–4174
22. Boscoboinik, D., Debanne, M. T., Stafford, A. R., Jung, C. Y., Gupta, R. S., and Epand, R. M. (1999) Biochim. Biophys. Acta 1327, 229–238
23. Jette, L., Potier, M., and Beliveau, R. (1997) Biochemistry 36, 13929–13937
24. Peruchynsky, M. S., and Ling, V. (1994) Biochemistry 33, 4163–4174
25. Boscoboinik, D., Debanne, M. T., Stafford, A. R., Jung, C. Y., Gupta, R. S., and Epand, R. M. (1999) Biochim. Biophys. Acta 1327, 229–238
26. Jette, L., Potier, M., and Beliveau, R. (1997) Biochemistry 36, 13929–13937
27. Peruchynsky, M. S., and Ling, V. (1994) Biochemistry 33, 4163–4174
28. Boscoboinik, D., Debanne, M. T., Stafford, A. R., Jung, C. Y., Gupta, R. S., and Epand, R. M. (1999) Biochim. Biophys. Acta 1327, 229–238
29. Jette, L., Potier, M., and Beliveau, R. (1997) Biochemistry 36, 13929–13937
30. Peruchynsky, M. S., and Ling, V. (1994) Biochemistry 33, 4163–4174
31. Boscoboinik, D., Debanne, M. T., Stafford, A. R., Jung, C. Y., Gupta, R. S., and Epand, R. M. (1999) Biochim. Biophys. Acta 1327, 229–238
32. Jette, L., Potier, M., and Beliveau, R. (1997) Biochemistry 36, 13929–13937
33. Peruchynsky, M. S., and Ling, V. (1994) Biochemistry 33, 4163–4174
34. Boscoboinik, D., Debanne, M. T., Stafford, A. R., Jung, C. Y., Gupta, R. S., and Epand, R. M. (1999) Biochim. Biophys. Acta 1327, 229–238
35. Jette, L., Potier, M., and Beliveau, R. (1997) Biochemistry 36, 13929–13937
36. Peruchynsky, M. S., and Ling, V. (1994) Biochemistry 33, 4163–4174

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