Oligomerization Domain of the Multidrug Resistance–Associated Transporter ABCG2 and Its Dominant Inhibitory Activity

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Abstract

Overexpression of human ATP-binding cassette transporter ABCG2 in cancer cells causes multidrug resistance by effluxing anticancer drugs. ABCG2 is considered as a half transporter and is thought to function as a homodimer. However, recent evidence suggests that it may exist as a higher form of oligomer consisting of 12 subunits. In this study, we mapped the oligomerization domain of human ABCG2 to its transmembrane domain consisting of TM5-loop-TM6. This oligomerization domain, when expressed alone in HEK293 cells, also forms a homododecamer. Furthermore, this domain has activity that inhibits drug efflux and resistance function of the full-length ABCG2 likely by disrupting the formation of the homo-oligomeric full-length ABCG2. These findings suggest that human ABCG2 may exist and work as a homo-oligomer by interactions located in TM5-loop-TM6, and that ABCG2 oligomerization may be used as a target for therapeutic development to circumvent ABCG2-mediated drug resistance in cancer treatment. [Cancer Res 2007;67(9):4373–81]

Introduction

Multidrug resistance is a serious problem in cancer chemotherapy. Overexpression of some members of the ATP-binding cassette (ABC) transporter superfamily causes increased efflux of anticancer drugs, resulting in decreased intracellular accumulation of anticancer drugs and, thus, the cancer cells can survive drug treatment (1–5). ABCG2 is one example of these transporters that have been shown to cause increased drug efflux and resistance (6–9).

Unlike traditional full ABC transporters with two transmembrane domains (TMD) and two nucleotide binding domains (NBD), ABCG2 consists of only one NBD and one TMD, with a domain structure of NBD-TMD. It has been thought to exist and function as a homodimer covalently linked by disulfide bonds (10–12) in the third extracellular loop between TM5 and TM6 (13, 14). However, it has been found recently that human ABCG2 exists in the drug-resistant cells mostly as a higher form of oligomer containing 12 subunits with noncovalent interactions (15).

In this study, we delineated the oligomerization domain of human ABCG2 and tested whether oligomerization could be exploited as a target for intervention of ABCG2-mediated drug resistance. We found that the domain consisting of TM5-loop-TM6 is responsible for ABCG2 oligomerization. Ectopically expressed TM5-loop-TM6 domain exists as a dodecamer, and its coexpression inhibits ABCG2-mediated drug efflux and resistance. These findings suggest that human ABCG2 likely exists and works as a homo-oligomer by interactions located in TM5-loop-TM6, and that ABCG2 oligomerization may be used as a target for therapeutic development to circumvent ABCG2-mediated drug resistance in cancer therapy.

Materials and Methods

Materials. Bxp-21 against ABCG2, anti-Myc and anti-HA antibodies, and protein G-Sepharose 4B were purchased from ID Labs, Cell Signaling, Covance, and Santa Cruz Biotechnology, respectively. G418, hygromycin, LipofectAMINE/Opti-MEM transfection reagents were from Invitrogen. pTK-Hyg plasmid, perfluoro-octanoic acid (PFO), protease inhibitor cocktail tablets, disuccinimidy1 sulferate (DSS) were from Clontech, Oakwood Products, Roche Diagnostics, and Pierce Biotechnology, respectively. N,N-dimethyl formamide, mitoxantrone, etoposide (VP-16), 3-(4,5-dimethyl-1azol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Triton X-100 were obtained from Sigma. Enhanced chemiluminescence reagents, Superdex 200 HR column, thyroglobulin, ovalbumin, and RNase A were from Amersham/Pharmacia. Polyvinylidene difluoride membranes, protein concentration assay kit, and precast polyacrylamide gradient gels were from Bio-Rad. Cell culture media and reagents were obtained from either Invitrogen or Cambrex Bioscience. All other reagents of molecular biology grade were purchased from Sigma or Fisher.

Engineering of HA or Myc-tagged ABCG2 constructs. All truncated constructs were engineered by first performing a PCR to amplify the cDNA of interest using primers carrying BamHI or EcoRI site. The PCR products were then digested with BamHI and EcoRI and subsequently cloned into pcDNA3, resulting in final expression constructs. All final constructs were verified by double-strand DNA sequencing.

To engineer tagged NBD (ABCG2Myc-NBD) and TMD (ABCG2TMD-HA), we first analyzed the amino acid sequence of human ABCG2 and found a potential linker region between NBD and TMD, 78 amino acids downstream of the Walker B sequence similar as that in human ABCB1. We thus engineered these constructs by using this potential linker region as cleavage sites. ABCG2Myc-NBD was produced by PCR using a Myc tag-encoding forward primer containing a BamHI site and a reverse primer containing an EcoRI site, a Myc and an HA encoding reverse primer containing an EcoRI site and a stop codon, a BamHI site and a reverse primer containing a BamHI site and an EcoRI site, and an HA encoding reverse primer containing an EcoRI site and a stop codon. ABCG2Myc-TM1,2 and ABCG2Myc-TM1,4 were cloned in the same way as ABCG2Myc-NBD with a Myc tag-encoding forward primer containing a BamHI site: 5′-CCCGGATCCCGGGCATTTGAAAAGACTCATTCTCGAGAAGATTTTTTCATCTTAGGAATTTTTG¬TTAAAATCAGATACAGGT-3′ (for ABCG2Myc-TM1,2) and 5′-CCCGGATCCCGGGCATTTGAAAAGACTCATTCTCGAGAATTTTTTCATCTTAGGAATTTTTG¬TTAAAATCAGATACAGGT-3′ (for ABCG2Myc-TM1,4).
ABCG2\textsuperscript{Myc-TMD-6} was engineered using PCR with a Myc-tag–encoding forward primer containing a BamHI site 5'-CCGCGATCCGCGCCATG-GACAAAAAGCTCTCTAGAAGAAGATCTGGCAGCAGGTCAGAGCTTGTTTGCTGTA-3' and a reverse primer containing an EcoRI site 5'-CCGGAATTCATTAAAGATTTTAAAGAATAAC-3'. To engineer HA- and Myc-tagged full-length ABCG2s (ABCG2\textsuperscript{HA-F} and ABCG2\textsuperscript{Myc-F}), cDNA fragments encoding the HA-tagged and Myc-tagged NH\textsubscript{2} termini were released from ABCG2\textsuperscript{HA-NBD} and ABCG2\textsuperscript{Myc-TMD-4} constructs using BamHI and AclI double digestion. These fragments were then used to replace the corresponding wild-type full-length ABCG2 sequence without tags in pcDNA3 to generate ABCG2\textsuperscript{HA-F} and ABCG2\textsuperscript{Myc-F}.

Cell culture and transfection. HEK293 cells were maintained at 37°C in 5% CO\textsubscript{2} in DMEM supplemented with 10% FCS. For transient transfections, cells at 90% confluency in 6-cm plate were transfected with 4 μg desired constructs using LipofectAMINE/Opti-MEM reagents. To select stable clones, the transfected cells were grown in the presence of 0.8 mg/mL G418. The stable clones were maintained in 0.2 mg/mL G418. To establish stable clones with expression of two different constructs, the stable clones selected by G418 were cotransfected with 2 μg pTK-Hyg, together with 20 μg desired constructs followed by selection using 0.2 mg/mL hygromycin. The positive double stable cell lines were maintained in 0.2 mg/mL G418 and 0.1 mg/mL hygromycin.

Cell membrane and lysate preparations. Plasma membranes were prepared in exactly the same way as previously described (15, 16), and the final membranes were resuspended in STBS [250 mM NaCl, 150 mM L-glutamic acid, 10 mM NaCl, 25 mM Tris–HCl (pH 7.4)] for lysate preparation, cells at 90% confluency were harvested and lysed in ice-cold lysis buffer [150 mM NaCl, 25 mM Tris (pH 7.4), 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100]. After 30 min incubation at 4°C followed by brief sonication, the lysates were cleared by centrifugation at 16,000 × g for 30 min. The protein concentrations of the membranes and lysates were determined using the Bio-Rad protein assay kit.

Immunoprecipitation. About 400 μg cell lysates were diluted to 1.0 mL using the same lysis buffer (see above), then mixed with 10 μL normal mouse immunoglobulin G (IgG), and incubated for 2 h at 4°C, followed by addition of 40 μL Protein G-Sepharose beads and further incubation for 2 h at 4°C. The mixture was then centrifuged at 500 × g for 1 min, the supernatants were transferred to fresh tubes and incubated with primary antibodies (anti-HA, anti-Myc, or control IgG, 1:100 dilution) for ≥5 h at 4°C. The reaction was centrifuged again at top speed for 15 min at 4°C, and the supernatants were transferred to fresh tubes and mixed with 40 μL Protein G-Sepharose beads and incubated overnight at 4°C with shaking. The immunoprecipitates were collected by centrifugation, washed five times with 1 mL lysis buffer each, and finally solubilized in 40 μL SDS sample buffer for Western blot analyses as previously described (15).

Immunofluorescence staining and confocal imaging. This experiment was done as previously described (17). Briefly, 5 × 10\textsuperscript{5} cells were cultured for 2 days, washed twice with PBS, fixed with 0.5 mL prechilled acetone/methanol (50:50, v/v) at room temperature for 10 min, and then blocked with ice-cold washing buffer (1% bovine serum albumin in PBS) for 30 min, followed by staining with anti-Myc antibody at 1:50 dilution for 1 h on ice. The cells were then washed twice with ice-cold washing buffer and incubated with FITC-conjugated anti-mouse IgG at 1:100 dilution, together with propidium iodide at 1:200 dilution for 1 h on ice. The staining was imaged using a Zeiss confocal microscope.

PFO-PAGE, nondenaturing PAGE, gel filtration chromatography, chemical cross-linking, and metabolic labeling. These experiments were done in exactly the same way as previously described (15). The stoichiometry of the heterocomplex was calculated using the following formula: Mr\textsubscript{complex} = N\textsubscript{Myc-TMD-6} × Mr\textsubscript{HA-F} + N\textsubscript{Myc-NBD} × Mr\textsubscript{Myc-NBD} + N\textsubscript{Myc-TMD-6} × N\textsubscript{HA-F} × Mr\textsubscript{HA-F} and N\textsubscript{Myc-TMD-6} + N\textsubscript{HA-F} = 12, where Mr represents apparent molecular weight, and N represents the numbers of subunits in the heterocomplex.

Drug efflux assay. Drug efflux assay was done as described previously (18) with some modifications. Briefly, 5 × 10\textsuperscript{5} cells were trypsinized and washed...
with PBS, resuspended in 0.5 mL PBS containing 20 μmol/L mitoxantrone, and incubated at 37°C for 30 min. Cells were then collected by centrifugation, washed twice with PBS, and resuspended in 0.5 mL PBS and incubated at 37°C for 1 h. The cells were then washed twice with PBS and analyzed by flow cytometry using a Becton Dickinson FACS calibur. The data were analyzed using Cell Quest Pro (BD Biosciences). In the negative vehicle controls, equal volumes of ethanol, which were used to dissolve mitoxantrone, were used.

**Cytotoxicity assay.** The cytotoxicity was measured using MTT and colony formation assays. MTT assay was done as previously described (19) using different concentrations of mitoxantrone and VP-16. EC50 is defined as the concentration of drugs required to kill 50% of the cells in the control condition without any drugs. Relative resistance factors were determined by dividing median EC50 of stable clones with expression of ABCG2ΔNBD-TM5-6 (TM5-6/Vec.), ABCG2ΔNBD-TM1-2 (Vec./TM1-2), or ABCG2ΔNBD-TM3-6 (lanes 4) were separated by SDS-PAGE followed by Western blot probed with anti-Myc, anti-HA, or anti-β-actin antibodies. Colony formation assay was done as described by Cheunget al. (20) using 17.5 nmol/L mitoxantrone or 340 nmol/L VP-16. Efficiency of colony formation was calculated by normalizing to the control treated with vehicles (ethanol for mitoxantrone and DMSO for VP-16).

**Results**

The ABCG2 oligomerization domain is located within the domain consisting of TM5-loop-TM6. To map the oligomerization domain of human ABCG2, we first tested if this putative domain is located in the amino terminal NBD or the carboxyl terminal TMD. For this purpose, we established HEK293 cell lines with stable expression of HA- or Myc-tagged full-length ABCG2 (ABCG2HA-F or ABCG2Myc-F; see Fig. 1A for constructs). We next generated two constructs encoding Myc-tagged NBD with residues M1-Y336 (ABCG2Myc-NBD) and HA-tagged TMD with residues K326-S655 (ABCG2TMD-HA; Fig. 1A) and transiently transfected them into the stable cell lines expressing ABCG2HA-F and ABCG2Myc-F, respectively. Lysates from these cells were first tested for coexpression of the full-length and half ABCG2 molecules using Western blot analysis. All constructs were well expressed in HEK293 cells (Fig. 1B). However, ABCG2TMD-HA seemed to be smaller than the expected size likely due to its high hydrophobicity. To rule out the possibility that the observed smaller size of ABCG2TMD-HA was due to truncation or internal translation initiation, we engineered and tested another construct double tagged with Myc and HA at its amino and carboxyl termini, respectively (Fig. 1A). As shown in Fig. 1C, the double-tagged TMD had similar mobility as the original HA-tagged TMD. Thus, the smaller observed size of TMD is not due to internal initiation or

**Figure 2.** Localization of ABCG2 oligomerization domain to TM5-loop-TM6. A, schematic structure of tagged full-length and deletion constructs. The symbol representations are the same as in Fig. 1A. B, Western blot analyses. Lysates (80 μg) from cells with stable expression of ABCG2ΔNBD-TM5-6 (lanes 1), ABCG2Myc-TM1-4 (lane 2), ABCG2Myc-TM3-6 (lane 3), or ABCG2Myc-TM5-6 (lane 4) were separated by SDS-PAGE followed by Western blot probed with anti-Myc, anti-HA, or anti-β-actin antibodies. C, confocal immunofluorescence imaging. HEK293 cells transfected with vector alone, ABCG2ΔNBD-TM5-6, or ABCG2ΔNBD-TM5-6 were stained with anti-Myc antibody followed by FITC-conjugated anti-mouse IgG. The nucleus was counterstained with propidium iodide. D, coimmunoprecipitation analyses. Lysates (400 μg; same as in B) were immunoprecipitated with anti-HA or anti-Myc antibodies followed by Western blot probed with the same two antibodies. Lysates from cells with stable ABCG2HA-F expression and transiently transfected with vector were also used as a negative control.
truncation. It is possible that more SDS is bound to the TMD due to its higher hydrophobicity, which would cause a faster mobility of the TMD on SDS-PAGE.

We next did coimmunoprecipitation and Western blot analyses of lysates from cells coexpressing full-length and domain constructs of ABCG2 using monoclonal anti-HA and anti-Myc antibodies. As shown in Fig. 1D and E, ABCG2Myc-NBD did not coimmunoprecipitate ABCG2HA-F (Fig. 1D), whereas ABCG2TMD-HA coimmunoprecipitated ABCG2Myc-F (Fig. 1E). Thus, it is likely that the oligomerization domain of ABCG2 is located within the TMD but not in the NBD.

To further map the oligomerization domain, we engineered two Myc-tagged constructs with sequential deletions of TM segments (ABCG2Myc-TM1-4 with residues M^1-V^536 and ABCG2Myc-TM1-2 with M^1-T^482; Fig. 2A; see also Fig. 1A). These constructs, together with ABCG2Myc-F as a control, were transiently transfected into HEK293 cells with stable expression of ABCG2Myc-F. Western blot analyses of cell lysates (Fig. 2B) and immunofluorescence staining of whole cells (Fig. 2C) were done to ensure the coexpression and proper trafficking to membranes of the truncated ABCG2s, respectively. It is noteworthy that ABCG2Myc-TM1-4 and ABCG2Myc-TM1-2 have similar apparent mobility on SDS-PAGE despite the fact that they have a difference of 54 amino acids in length. This may be again due to the fact that the additional 54 amino acids (TM3 and TM4) at the carboxyl terminus of ABCG2Myc-TM1-4 (see Fig. 2A) are mostly hydrophobic residues, which may cause increased mobility and a smaller apparent molecular weight than its true size, similar to the one observed with TMD discussed above in Fig. 1. The use of high concentration gel may also contribute to the less clear difference between the two proteins. We next did coimmunoprecipitation and Western blot analyses with anti-HA and anti-Myc antibodies. As shown in Fig. 2D, ABCG2Myc-F coprecipitated ABCG2HA-F and

\begin{figure}
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\includegraphics[width=\textwidth]{figure3}
\caption{Characterization of ABCG2Myc-TM5-6 complex. A, gel filtration chromatography. About 180 \textmu g membranes from HEK293 cells with stable ABCG2Myc-TM5-6 expression were extracted using 1% SDS (top) or 0.5% PFO (bottom), and the extract was separated by gel filtration chromatography using Superdex column followed by TCA precipitation, SDS-PAGE, and Western blot analyses. The markers used were also treated and separated the same way and detected by a UV detector. The size of ABCG2Myc-TM5-6 was estimated based on linear regression of protein markers: TG, thyroglobulin (669 kDa); BSA, bovine serum albumin (67 kDa); OA, ovalbumin (43 kDa); CT, chymotrypsinogen A (25 kDa); RA, RNase A (14 kDa); AU, arbitrary unit. B, PFO- and nondenaturing PAGE. Membranes (50 \textmu g) from HEK293 cells with stable ABCG2Myc-TM5-6 expression were extracted using 0.5% PFO or 1% Triton X-100 and then separated by PFO- (lane 1) or nondenaturing PAGE (lane 2), followed by Western blot analysis. C, chemical cross-linking. HEK293 cells with stable ABCG2Myc-TM5-6 expression were first treated without (–) or with (+) 2 mmol/L DSS, and crude membranes were isolated for SDS-PAGE and Western blot analyses. The size of ABCG2Myc-TM5-6 was estimated based on linear regression of the protein markers used. D, coimmunoprecipitation. HEK293 cells with stable ABCG2Myc-TM5-6 expression were metabolically labeled with [35S]methionine, followed by immunoprecipitation of lysate with anti-Myc or control IgG, separation on SDS-PAGE, and autoradiography to detect coprecipitated proteins. Myc antibody was used in all Western blot analyses.}
\end{figure}
Targeting ABCG2 Oligomerization for Chemosensitization

vice versa as expected, whereas neither ABCG2<sup>Myc-TM1-4</sup> nor ABCG2<sup>Myc-TM1-2</sup> coprecipitated ABCG2<sup>HA-F</sup>, suggesting that deletion of the domain consisting of TM5-loop-TM6 may have removed the oligomerization activity. It is thus possible that the oligomerization domain of human ABCG2 is located within TM5-loop-TM6.

To test this possibility, we engineered another truncated ABCG2 construct encoding only TM5-loop-TM6 (residues A<sup>528</sup>-S<sup>655</sup>) with a Myc tag (ABCG2<sup>Myc-TM5-6</sup>; Fig. 2A, also see Fig. 1A) and did similar experiments as described above. Figure 2B and C shows that ABCG2<sup>Myc-TM5-6</sup> could be successfully coexpressed with ABCG2<sup>HA-F</sup> and properly trafficked onto plasma membranes, respectively. ABCG2<sup>Myc-TM5-6</sup> could also coprecipitate ABCG2<sup>HA-F</sup> and vice versa (Fig. 2D). Thus, it is likely that the oligomerization domain of human ABCG2 is located within TM5-loop-TM6.

The TM5-loop-TM6 domain can form a homododecamer. To further determine if this domain indeed has oligomerization activity, we analyzed if it can form a homo-oligomer and estimated the number of subunits within the oligomer. For this purpose, a stable cell line overexpressing ABCG2<sup>Myc-TM5-6</sup> was established and subjected to several biochemical analyses to determine its native size.

Gel filtration chromatography (Superdex) was first employed to determine the oligomeric status of human ABCG2<sup>Myc-TM5-6</sup> following extraction from isolated plasma membranes with 0.5% PFO or 1% SDS as a control as previously described (15). The eluted fractions from gel filtration were trichloroacetic acid (TCA) precipitated, separated by SDS-PAGE, and followed by Western blot to detect ABCG2<sup>Myc-TM5-6</sup>. We found that ABCG2<sup>Myc-TM5-6</sup> extracted by SDS was eluted in the fractions with retention volume of 12 to 13.5 mL with an estimated average molecular weight of ~19 kDa (Fig. 3A, top; Table 1), close to that determined by SDS-PAGE (Fig. 3C and D and Table 1). ABCG2<sup>Myc-TM5-6</sup> extracted by PFO was eluted mainly in the fraction of a retention volume of ~9 mL with an estimated molecular weight of ~261 kDa (Fig. 3A, bottom; Table 1). Assuming that ABCG2<sup>Myc-TM5-6</sup> in SDS behaves as a monomer, the native ABCG2<sup>Myc-TM5-6</sup> in the peak fraction extracted by PFO is likely a dodecamer as we previously found for the full-length ABCG2 (15).

We next used PFO-PAGE followed by Western blot to detect ABCG2<sup>Myc-TM5-6</sup>. This method has been used successfully in the past for studying several membrane proteins, including native ABCG2 (15, 21–24). As shown in Fig. 3B (lane 1), ABCG2<sup>Myc-TM5-6</sup> extracted from isolated membranes using 0.5% PFO migrated as a broad band on PFO-PAGE, with an estimated average molecular weight of ~226 kDa (Table 1). We also did a nondenaturing PAGE and Western blot analysis of ABCG2<sup>Myc-TM5-6</sup> extracted by nonionic detergent Triton X-100 to rule out the potential problem with the use of PFO. As shown in Fig. 3B (lane 2), ABCG2<sup>Myc-TM5-6</sup> again migrated mainly with a mobility of ~235 kDa (Table 1). Given that ABCG2<sup>Myc-TM5-6</sup> in SDS has an apparent molecular weight of ~19 kDa in gel filtration and ~17 kDa on SDS-PAGE (see Table 1), ABCG2<sup>Myc-TM5-6</sup> separated by PFO- and nondenaturing PAGE likely represents dodecameric complexes. It should also be noted that isolated plasma membranes were used in the above studies, and the findings confirm our conclusion that ABCG2<sup>Myc-TM5-6</sup> is located on plasma membranes.

To further determine whether the dodecameric ABCG2<sup>Myc-TM5-6</sup> exists in live cells, we conducted a chemical cross-linking experiment of live cells using DSS as previously described (15). Membranes were then isolated and subjected to SDS-PAGE and Western blot analysis to detect ABCG2<sup>Myc-TM5-6</sup>. As shown in Fig. 3C, at least five cross-linked products were detected by anti-Myc antibody. The estimated molecular weights of these products are 179, 94, 62, 41, and 32 kDa, which likely correspond to dodecameric, hexameric, tetrameric, trimeric, and dimeric ABCG2<sup>Myc-TM5-6</sup>, respectively (see Table 1). The un–cross-linked monomeric ABCG2<sup>Myc-TM5-6</sup> has an apparent molecular weight of ~17 kDa on SDS-PAGE (Fig. 3C). It should be noted that the minor lower monomeric band (possibly a degradation product, see Discussion) disappeared following cross-linking. It is possible that it became undetectable following cross-linking due to its relatively low abundance and conversion to cross-linked molecules.

To test whether the oligomeric ABCG2<sup>Myc-TM5-6</sup> is homogeneous, we did immunoprecipitation assay following metabolic labeling with [35S]methionine of the cells with stable expression of ABCG2<sup>Myc-TM5-6</sup> and lysis of cells with Triton X-100. As shown in Fig. 3D, no additional proteins were coprecipitated with ABCG2<sup>Myc-TM5-6</sup>, suggesting that ABCG2<sup>Myc-TM5-6</sup> is likely a homooligomer.

Coexpression of the TM5-loop-TM6 domain inhibits ABCG2 functions. As shown above, ABCG2<sup>Myc-TM5-6</sup> is expressed on plasma membranes, contains the oligomerization domain, and can interact with the full-length ABCG2. It is of interest to determine if ABCG2<sup>Myc-TM5-6</sup> inhibits ABCG2 function by interacting with the full-length ABCG2 and competing for oligomerization. We first tested if coexpression of ABCG2<sup>Myc-TM5-6</sup> inhibits ABCG2-mediated drug resistance. For this purpose, stable cell clones expressing both

### Table 1. Summary of apparent molecular weight (Mr) of human ABCG2<sup>Myc-TM5-6</sup>

<table>
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<tr>
<th>Methods</th>
<th>Mr (kDa)</th>
<th>No. subunits</th>
<th>Oligomeric state</th>
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</thead>
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<td>Gel filtration (Superdex)</td>
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<tr>
<td>0.5% PFO</td>
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<td>15</td>
<td>1.0&lt;sup&gt;n&lt;/sup&gt;</td>
<td>Monomer</td>
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</table>

<sup>a</sup>The size used for calculation is 19 kDa determined from gel filtration chromatography in SDS using Superdex.

<sup>b</sup>The size used for calculation is 17 kDa determined from SDS-PAGE without the use of DSS.

<sup>c</sup>This cross–linked product has an estimated size of 179 kDa based on linear regression analysis of molecular weight markers, although it seemed to migrate slightly slower than the 182-kDa marker on the gel (see Fig. 3D).

<sup>d</sup>The size used for calculation is 15 kDa determined from gel filtration chromatography in SDS using Superose.

<sup>e</sup>The size used for calculation is 19 kDa determined from gel filtration chromatography in SDS using Superdex.

<sup>f</sup>The size used for calculation is 17 kDa determined from SDS-PAGE without the use of DSS.

<sup>g</sup>This cross–linked product has an estimated size of 179 kDa based on linear regression analysis of molecular weight markers, although it seemed to migrate slightly slower than the 182-kDa marker on the gel (see Fig. 3D).

<sup>h</sup>The size used for calculation is 15 kDa determined from gel filtration chromatography in SDS using Superose.

<sup>i</sup>The size used for calculation is 19 kDa determined from gel filtration chromatography in SDS using Superdex.

<sup>j</sup>The size used for calculation is 17 kDa determined from SDS-PAGE without the use of DSS.

<sup>k</sup>This cross–linked product has an estimated size of 179 kDa based on linear regression analysis of molecular weight markers, although it seemed to migrate slightly slower than the 182-kDa marker on the gel (see Fig. 3D).

<sup>l</sup>The size used for calculation is 15 kDa determined from gel filtration chromatography in SDS using Superose.

<sup>m</sup>The size used for calculation is 19 kDa determined from gel filtration chromatography in SDS using Superdex.
ABC\textsubscript{2}\textsuperscript{Myc-TM5-6} and ABC\textsubscript{2}\textsuperscript{HA-F} and vector control clones were first established using double selection as described in Materials and Methods. Figure 4A shows the expression of ABC\textsubscript{2}\textsuperscript{Myc-TM5-6} and ABC\textsubscript{2}\textsuperscript{HA-F}, together or alone with vector controls as determined by Western blot analysis. We next did drug resistance analysis using MTT assay with anticancer drugs mitoxantrone and VP-16. As shown in Fig. 4B, the cells expressing ABC\textsubscript{2}\textsuperscript{HA-F} alone (Vec./HA-F) are significantly more resistant to both drugs tested than the cells transfected with vector controls alone (Vec./Vec.) or cells expressing only ABC\textsubscript{2}\textsuperscript{Myc-TM5-6} (TM5-6/Vec.). However, the resistance to these drugs due to ABC\textsubscript{2}\textsuperscript{HA-F} expression was significantly decreased by coexpressing ABC\textsubscript{2}\textsuperscript{Myc-TM5-6} (TM5-6/Vec.) and ABC\textsubscript{2}\textsuperscript{HA-F}. These observations were further confirmed when colony formation assay was used. As shown in Fig. 4C, coexpression of ABC\textsubscript{2}\textsuperscript{Myc-TM5-6} significantly reduced the number of colonies formed by the resistant cells (Vec./HA-F) in the presence of anticancer drugs mitoxantrone or VP-16.

To determine if the inhibition of drug resistance by ABC\textsubscript{2}\textsuperscript{Myc-TM5-6} was due to its effect on ABC\textsubscript{2}-mediated drug efflux, we employed the established fluorescence-activated cell sorting (FACS) analysis for mitoxantrone efflux. We first transiently transfected 2 μg of the untagged full-length ABC\textsubscript{2} into HEK293 cells with stable expression of ABC\textsubscript{2}\textsuperscript{Myc-TM5-6}, followed by testing the drug efflux activity of the transiently transfected ABC\textsubscript{2} after transfection. Figure 5A shows the expression of ABC\textsubscript{2}\textsuperscript{Myc-TM5-6} and ABC\textsubscript{2}\textsuperscript{F} together or alone and control cells transfected with vectors as determined by Western blot analyses. Figure 5B shows typical profiles of mitoxantrone efflux in these cells as determined by FACS. As expected, the cells harboring vectors alone (Vec./Vec.) or expressing only ABC\textsubscript{2}\textsuperscript{Myc-TM5-6} (TM5-6/Vec.) have the highest level of mitoxantrone accumulation (less effluxed), whereas the cells expressing only ABC\textsubscript{2}\textsuperscript{F} (Vec./F) have the lowest level of drug accumulation (more effluxed). Interestingly, the cells expressing both ABC\textsubscript{2}\textsuperscript{Myc-TM5-6} and ABC\textsubscript{2}\textsuperscript{F} (TM5-6/F) have intermediate levels of mitoxantrone accumulation, suggesting that the mitoxantrone efflux activity of ABC\textsubscript{2}\textsuperscript{F} has been tampered by coexpressing ABC\textsubscript{2}\textsuperscript{Myc-TM5-6}, and thereby, the cells have more drugs accumulated compared with the cells expressing only ABC\textsubscript{2}\textsuperscript{F}. We also did another negative control experiment using ABC\textsubscript{2}\textsuperscript{Myc-TM1-4} and found that it, similar to the vector, had no effect on ABC\textsubscript{2}\textsuperscript{F}-mediated mitoxantrone efflux (data not shown). Thus, most likely, the effect of ABC\textsubscript{2}\textsuperscript{Myc-TM5-6} on ABC\textsubscript{2}-mediated drug efflux is due to its direct interaction with the full-length ABC\textsubscript{2}.

We next conducted an assay using cells transiently transfected with different amounts of ABC\textsubscript{2} cDNA into stable clones expressing ABC\textsubscript{2}\textsuperscript{Myc-TM5-6} to determine the dose response by elevating the ABC\textsubscript{2}\textsuperscript{F} expression level. We did not perform the transient transfection of ABC\textsubscript{2}\textsuperscript{Myc-TM5-6} into cells with stable expression of ABC\textsubscript{2}\textsuperscript{F} because of concerns that the efficiency of transient transfection of ABC\textsubscript{2}\textsuperscript{Myc-TM5-6} would be low, and many cells that have the stable expression of ABC\textsubscript{2}\textsuperscript{F} would not have been transfected by ABC\textsubscript{2}\textsuperscript{Myc-TM5-6} and, thus, affecting the outcome and interpretation. On the other hand, transfecting ABC\textsubscript{2}\textsuperscript{F} into cells with stable expression of ABC\textsubscript{2}\textsuperscript{Myc-TM5-6} will help ensure every cell that has ABC\textsubscript{2}\textsuperscript{F} will also have ABC\textsubscript{2}\textsuperscript{Myc-TM5-6}. Figure 5C shows the differential expression of ABC\textsubscript{2}\textsuperscript{F} in stable clones harboring vector control (lane 1) or ABC\textsubscript{2}\textsuperscript{Myc-TM5-6} (lanes 2–4). Figure 5D shows that 10 μg ABC\textsubscript{2}\textsuperscript{F}-transfected cells in the presence of ABC\textsubscript{2}\textsuperscript{Myc-TM5-6} [TM5-6/F(10)] had only the same level of efflux activity compared with the 2-μg ABC\textsubscript{2}\textsuperscript{F}-transfected cells in the absence of ABC\textsubscript{2}\textsuperscript{Myc-TM5-6} [Vec/F(2)], although the former had ~3-fold ABC\textsubscript{2}\textsuperscript{F}, suggesting that the efflux activity of ABC\textsubscript{2}\textsuperscript{F} was severely inhibited by coexpressing ABC\textsubscript{2}\textsuperscript{Myc-TM5-6}. Hence, we conclude that the domain consisting of TM5-loop-TM6 can inhibit the drug efflux and resistance function of ABC\textsubscript{2}, and that this inhibition is likely dose-dependent.

**TM5-loop-TM6 domain competes for oligomerization with full-length ABC\textsubscript{2}**. We have shown above that the coexpression of the TM5-loop-TM6 domain inhibits the function of ABC\textsubscript{2} (Figs. 4 and 5), and that it interacts with ABC\textsubscript{2} by coimmunoprecipitation (Fig. 3). To further determine if the coexpression of TM5-loop-TM6 domain affects the homooligomerization of full-length ABC\textsubscript{2}, we characterized hetero-oligomers in the stable cell clone that coexpresses both ABC\textsubscript{2}\textsuperscript{Myc-TM5-6} and ABC\textsubscript{2}\textsuperscript{HA-F} using gel filtration chromatography (Superose) as described previously (15).

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**Figure 4.** Inhibitory effect of ABC\textsubscript{2}\textsuperscript{Myc-TM5-6} on drug resistance of full-length ABC\textsubscript{2}. HEK293 cells with stable expression of both ABC\textsubscript{2}\textsuperscript{Myc-TM5-6} and ABC\textsubscript{2}\textsuperscript{HA-F} together (Myc-TM5-6/HA-F) or alone (Myc-TM5-6/Vec. and Vec./HA-F) and control vectors (Vec./Vec.) were subjected to Western blot analyses of lysates for expression (A), MTT assay for relative resistance factors to mitoxantrone and VP-16 (B), and colony formation assay (C).
Figure 5. Inhibitory effect of ABCG2<sup>My-TM5-6</sup> on drug efflux activity of full-length ABCG2. HEK293 cells stably transfected with vector or ABCG2<sup>My-TM5-6</sup> were subjected to transient transfection with vector and various amounts of full-length ABCG2, followed by Western blot analyses of cell lysate for expression (A and C) or FACs analysis of mitoxantrone efflux for transport activity of ABCG2 (B and D).

As shown in Fig. 6A, ABCG2<sup>HA-F</sup> alone in Triton X-100 was eluted in fractions of 10.5 to 13.5 mL, with an estimated average molecular weight of ~854 kDa, which corresponds to dodecamer, consistent with our previous findings (15). When coexpressed with ABCG2<sup>My-TM5-6</sup> in a stable cell clone, the elution of ABCG2<sup>HA-F</sup> shifted to the right in fractions of 12 to 14 mL, with an estimated average molecular weight of ~476 kDa (Fig. 6B). This observation suggests that the ABCG2<sup>HA-F</sup> complex in the presence of ABCG2<sup>My-TM5-6</sup> likely contains the smaller ABCG2<sup>My-TM5-6</sup> subunit. This was confirmed by testing these fractions with myc antibody for the coexistence of ABCG2<sup>My-TM5-6</sup>. As shown in Fig. 6C, most coexpressed ABCG2<sup>My-TM5-6</sup> was separated in fractions of 13 to 14 mL, with an average molecular weight of ~371 kDa. However, ABCG2<sup>My-TM5-6</sup> alone was eluted mainly with retention of 14.5 to 15.5 mL, with an average molecular weight of ~148 kDa (Fig. 6D).

Based on these observations, we conclude that coexpression of ABCG2<sup>HA-F</sup> and ABCG2<sup>My-TM5-6</sup> generated hetero-complexes consisting of both ABCG2<sup>HA-F</sup> and ABCG2<sup>My-TM5-6</sup>. Using the estimated molecular weight of ~15 kDa for ABCG2<sup>HA-TM5-6</sup> separated by Superose in SDS (Table 1), the estimated ABCG2<sup>HA-F</sup> molecular weight of 72 kDa (25) and the estimated average molecular weight of 371 kDa for the heterocomplex (Fig. 6C and Table 1), we calculated that the average stoichiometry of the heterocomplexes is 3 ABCG2<sup>HA-F</sup> + 9 ABCG2<sup>My-TM5-6</sup>, with a range from 5 ABCG2<sup>HA-F</sup> + 7 ABCG2<sup>My-TM5-6</sup> (Fig. 6C, fraction 13 mL) to 2 ABCG2<sup>HA-F</sup> + 10 ABCG2<sup>My-TM5-6</sup> (Fig. 6C, fraction 14 mL).

**Discussion**

ABCG2 is an important drug efflux pump causing drug resistance when overexpressed in cancer cells. It has been suggested that ABCG2 exists as a homodimer and possibly as a homododecamer. However, the oligomerization domain remained elusive. In this study, we have successfully mapped the oligomerization domain of ABCG2 to TM5-loop-TM6 (ABCG2<sup>My-TM5-6</sup>) and showed that ABCG2<sup>My-TM5-6</sup> alone can form a homododecamer, and it can inhibit the drug efflux and resistance function of human ABCG2.

Although each method generated slightly different results, the estimated size of domain ABCG2<sup>My-TM5-6</sup> complex was very large (148–261 kDa; see Table 1), and the complex was estimated to consist of 10 to 14 subunits calculated based on the size of monomeric proteins determined in each respective method. The highest oligomeric form that can be chemically cross-linked had an apparent size of ~179 kDa. Considering that the oligomeric ABCG2<sup>My-TM5-6</sup> seemed to be homogeneous (Fig. 3D) and that the size determination by these methods is only an estimate, we conclude that the ABCG2<sup>My-TM5-6</sup> may be a homododecamer consisting of 12 ABCG2<sup>My-TM5-6</sup> subunits. This conclusion further supports that human ABCG2 is likely a homododecamer and confirms that the interactions between ABCG2 subunits are likely located within the domain containing TM5-loop-TM6.

We also observed that ABCG2<sup>My-TM5-6</sup> seems to be a doublet on SDS-PAGE in most cases, especially when expressed alone (see Fig. 6D). The doublet disappears in other cases, especially when it is coexpressed with the full-length ABCG2 (see Fig. 6C). The reason for these differences is currently unknown. It is, however, possible that the oligomerization with the full-length molecule may help the maturation and stabilization of ABCG2<sup>My-TM5-6</sup>.

Recently, the intermolecular disulfide bond of ABCG2 has been localized to the extracellular loop (Cys<sup>605</sup>) linking TM5 and TM6 (13, 14), suggesting that this loop is likely involved in intersubunit interactions. Our finding, that the domain including TM5-loop-TM6 contains oligomerization activity, is consistent with these
previous observations. However, because the intermolecular disulfide bond is only for the dimeric formation as previously shown (13, 14), the finding that ABCG2Myc-TM5-6 could form a homododecamer suggests that the domain containing TM5-loop-TM6 is likely also involved in other types of noncovalent protein-protein interactions. Indeed, it has recently been found that a Gly residue (G553) in TM5 is potentially involved in ABCG2 oligomerization (26).

The GXXG motif in transmembrane segments has been shown responsible for oligomerization of membrane proteins (27, 28), and such a motif has been found in TM1, but not in TM5 and TM6, of ABCG2. However, removal of this motif in TM1 by site-directed mutagenesis did not abolish oligomerization of ABCG2, suggesting that the GXXG motif in TM1 does not play an essential role in ABCG2 oligomerization (29). This conclusion is supported by our current finding that the truncated ABCG2 containing TM1 to TM4 (ABCG2Myc-TM1-4) did not contain an oligomerization domain (Fig. 2).

Several other motifs, such as AXXXA, AXXGX, GXXXXXG, and QXXX, have been suggested to involve interactions between membrane proteins (28, 30, 31). Examination of the sequence of the domain containing TM5-loop-TM6 of human ABCG2 revealed that they contain only the QXXS motif (Q568YFS) in the loop linking TM5 and TM6. This sequence is completely conserved between the human and mouse ABCG2s. Examination of other members of the ABCG subfamily showed that human ABCG1 (Q594WMS) and ABCG4 (Q563WSS) and mouse ABCG1 (Q583WMS) and ABCG4 (Q563WSS) all have the QXXS motif in their loops linking TM5 and TM6. However, human ABCG5 and ABCG8 were found to have only Q568KYCS and Q607FS in their loops linking TM5 and TM6, although both have been shown to form heterodimers (32). Whether the QXXS motif is involved in oligomerization of ABCG2 and other ABCG subfamily members and whether the semi-conserved motif Q568KYCS and Q607FS in ABCG5 and ABCG8 are important for their heterodimerization await further investigations.

We also found that ABCG2Myc-TM5-6 has an inhibitory effect on the function of the full-length human ABCG2 in both drug resistance and efflux. This effect may be due to the incorporation of ABCG2Myc-TM5-6 into the complex by competing for ABCG2 subunits. This finding also suggests that the oligomerization of ABCG2 may be used as a target to develop therapeutics for treating drug-resistant human cancers. Small molecules that mimic the oligomerization domain and are able to disrupt oligomerization of ABCG2 may be developed as therapeutics in the future to circumvent ABCG2-mediated drug resistance in cancer treatment.

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References

Oligomerization Domain of the Multidrug Resistance–Associated Transporter ABCG2 and Its Dominant Inhibitory Activity

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