Identification of Intra- and Intermolecular Disulfide Bridges in the Multidrug Resistance Transporter ABCG2*

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ABCG2 is an ATP binding cassette (ABC) half-transporter that plays a key role in multidrug resistance to chemotherapy. ABCG2 is believed to be a functional homodimer that has been proposed to be linked by disulfide bridges. We have investigated the structural and functional role of the only three cysteines predicted to be on the extracellular face of ABCG2. Upon mutation of Cys-592 or Cys-608 to alanine (C592A and C608A), ABCG2 migrated as a dimer in SDS-PAGE under non-reducing conditions; however, mutation of Cys-603 to Ala (C603A) caused the transporter to migrate as a single monomeric band. Despite this change, C603A displayed efficient membrane targeting and preserved transport function. Because the transporter migrated as a dimer in SDS-PAGE, when only Cys-603 was present (C592A-C608A), the data suggest that Cys-603 forms a symmetrical intermolecular disulfide bridge in the ABCG2 homodimer that is not essential for protein expression and function. In contrast to C603A, both C592A and C608A displayed impaired membrane targeting and function. Moreover, when only Cys-592 or Cys-608 were present (C592A/C603A and C603A/C608A), the transporter displayed impaired plasma membrane expression and function. The combined mutation (C592A/C608A) partially restored plasma membrane expression; however, although transport of mitoxantrone was almost normal, we observed impairment of BODIPY-prazosin transport. This supports the conclusion that Cys-592 and Cys-608 form an intramolecular disulfide bridge in ABCG2 that is critical for substrate specificity. Finally, mutation of all three cysteines simultaneously resulted in low expression and no measurable function. Altogether, our data are consistent with a scenario in which an inter- and an intramolecular disulfide bridge together are of fundamental importance for the structural and functional integrity of ABCG2.

Multidrug resistance represents a serious problem in cancer chemotherapy. The resistant tumor cells often overexpress one of several ATP binding cassette (ABC) transporters that are capable of mediating efflux of clinically important drugs (1). One of these is ABCG2, also known as MXR, ABCP, and BCRP (2–4). ABCG2 is expressed in many different cancer tissues (5) and several different types of leukemia (6, 7).

ABCG2 expression is up-regulated particularly in cells exposed to mitoxantrone, a drug often used in the treatment of breast cancer (8). ABCG2 is situated in the plasma membrane (9) where it mediates efflux not only of mitoxantrone but also of flavopiridol, camptothecins, and methotrexate (10–13). ABCG2 has the highest expression level in the placenta and is also expressed in many other tissues, such as the ducts and lobules of the breast, in the small and large intestine, and the canalicular membrane of the liver (14). Although a physiological function of ABCG2 remains to be established, the tissue distribution suggests a role of the protein in the protection against xenobiotics. Lately, ABCG2 has been found in relation to stem cells (15, 16) and sterol transport (17).

ABCG2 is a half-transporter with an N-terminal nucleotide binding domain and a C-terminal transmembrane domain. Many ABC half-transporters function as homo- or heterodimers (18, 19), and it is widely accepted that ABCG2 exists as a homodimer (20–22). It has, however, also been proposed that ABCG2 exists in the cell as a homotetramer (23). Our previous studies have supported that ABCG2 is indeed an oligomer (most likely a homodimer), and function of the transporter is dependent on this dimerization (24). However, our previous data also shows that dimerization of the transporter is not dependent on functional nucleotide binding domains, as an inactivating mutation (K86M) in the Walker A motif did not alter the ability of ABCG2 to form dimers (24). Hence, the molecular basis responsible for dimerization of ABCG2 remains to be clarified. It was recently proposed that a known dimerization motif, the GXXXG motif (25, 26), located in TM1 of ABCG2 is important for proper protein processing and function, but it was also found not to be the sole mechanism of dimerization (27).

In our previous study, we present evidence that ABCG2 most likely is a disulfide bridge-linked dimer (24), and this has also been proposed by others (20, 21). According to the predicted topology of ABCG2, a large 68-residue-long extracellular loop exists between TM5 and TM6 (see Fig. 1). Interestingly, there are three cysteine residues in this loop in close proximity to each other, i.e. Cys-592, Cys-603, and Cys-608 (Fig. 1). In this study, we have carried out a systematic mutagenesis analysis of these cysteines to assess their role in the structure and covalent linking of the ABCG2 homodimer.

MATERIALS AND METHODS

Plasmids, Drugs, and Antibodies—pcDNA3.1(−)MXR (R482G) and fumitremorgin C (FTC) was kindly provided by Dr. Susan Bates, NCI, National Institutes of Health. BODIPY-prazosin was obtained from Molecular Probes. Biocytine-maleimide came from Invitrogen. TCEP, sodium-2-mercapto-ethanesulfonate, and all other chemicals were obtained from Sigma. The following antibodies were used: anti-ABCG2 (SD3, eBioscience, San Diego, CA), BXP-21 (Alexis Biochemicals, Montreal, Canada) and Alexa Fluor® 488 goat anti-mouse (Molecular Probes, Eugene, OR).
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Construction of Mutations—The ABCG2 cysteine mutations were generated by a two-generation PCR technique using the Pfu polymerase (Stratagene, La Jolla, CA). The generated PCR fragments were digested with the appropriate enzymes, purified by agarose gel electrophoresis, and cloned into pCIN4, a bicistronic mammalian expression vector that confers geneticin (G418) resistance (28). All constructs were confirmed and cloned into pCIN4, a bicistronic mammalian expression vector that

Cell Culture and Stable Transfection—HEK293 cells were maintained at 37 °C in 5% CO₂ in Dulbecco’s modified Eagle’s medium with Glutamax I supplemented with 10% fetal calf serum and 0.01 mg/ml gentamycin (all products from Invitrogen). For stable transfections, 1 × 10⁶ HEK293 cells were seeded in 75-cm² flasks. The cells were transfected with 5 μg of the pCIN4 constructs using the Lipofectamine™/Opti-MEM™ (Invitrogen) transfection system. A stably transfected pool clone was selected using G418 (0.35 mg/ml) and maintained in 0.2 mg/ml G418.

Total Cell Lysates and Western Blotting—Cells were grown to 90% confluence in 6-well plates and harvested in solubilization buffer (150 mM NaCl, 25 mM Tris (pH 7.4), 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 5 mM N-ethylmaleimide (NEM), 1% Triton X-100 and protease inhibitors) while kept on ice. After 30 min of incubation with end-over-end rotation at 4 °C, the lysates were cleared by centrifugation at 16000 × g for 30 min, and protein concentration was measured. Protein samples were added 2× SDS loading buffer (± 100 mM DTT, +10 mM NEM) and incubated for 30 min at 37 °C. The samples were applied onto SDS-polyacrylamide gels, blotted onto a polyvinylidene difluoride membrane and incubated in blocking buffer (TST, 10 mM Tris-HCl, 2.5 mM EDTA, 100 mM NaCl, and 0.1% Tween 20 + 5% dry milk (w/v)). Primary antibody was 1:1000 BXP-21, and secondary antibody was 1:10000 horseradish peroxidase-conjugated goat-anti-mouse antibody. The blots were developed using Supersignal chemiluminescent substrate (Pierce).

Cytotoxicity Assay—The cytotoxicity assay was performed as described by Skehan et al. (29). Stably transfected HEK293 cells were seeded (2000 cells/well) in sterile 96-well microtiter plates (Costar, Corning, Acton, MA) in 100 μl of Dulbecco’s modified Eagle’s medium with Glutamax and incubated 24 h at 37 °C. Mitoxantrone was added in 10 different final concentrations in 100 μl of Dulbecco’s modified Eagle’s medium. After incubation for 72 h at 37 °C, the cells were fixed in 50% trichloroacetic acid. The fixed cells were stained with 100 μl of 0.4% sulforhodamine B (SRB) in 1% acetic acid. Finally, the SRB dye was dissolved in 10 mM unbuffered Tris base (pH 10.5) for 10 min and measured at A₅₆₂ in a microplate reader (Bio-Tek Instruments, Winooski, VT).

Efflux Assay—The efflux assay was performed as described by Lee et al. 1994 (30). One 175-cm² flask of stably transfected HEK293 cells were harvested, resuspended in 10 ml of improved modified Eagle’s medium, and washed once. During the accumulation period, 3 aliquots of cells were suspended in 100 μl of improved modified Eagle’s medium, containing solvent (i), BODIPY-prazosin (100 nM) (ii), or BODIPY-prazosin (100 nM) + 5 μM FTC (iii) and incubated for 30 min at 37 °C. The assay was similarly performed with 10 μM mitoxantrone dissolved in water. After incubation, the cells were centrifuged for 3 min at 1000 × g. The samples were washed in PBS and resuspended in improved modified Eagle’s medium, without (ii) or with (iii) 5 μM FTC and incubated for 60 min at 37 °C. Following incubation, the samples were washed in improved modified Eagle’s medium, resuspended in 0.5 ml of PBS, and placed on ice in the dark. Finally, the samples were analyzed by flow cytometry using a BD Biosciences FACScalibur flow cytometer, and data were analyzed in Cell Quest Pro (BD Biosciences).
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RESULTS

We have previously proposed that ABCG2 is a structural and functional homodimer and that this dimer is disrupted by the addition of DTT in the loading buffer prior to analysis by SDS-PAGE (24). As shown in Fig. 2, ABCG2-wt migrates on SDS-PAGE as a dimer (~160 kDa) in the absence of DTT and as a monomer (~75 kDa) in the presence of 100 mM DTT. To exclude the possibility that any disulfide bridges irrelevant for the native structure of the protein were formed during the extraction procedure, high concentrations of the sulphydrol alkylating reagent NEM was added both to the lysis buffer (5 mM) and to the SDS-polyacrylamide gel loading buffer (10 mM).

Based on the predicted topology of ABCG2, there are three cysteines in the large extracellular loop connecting TM5 and TM6: Cys-592, Cys-603, and Cys-608 (Fig. 1). To analyze the influence of each of these putative extracellular cysteines on ABCG2 dimerization, we substituted, one by one, the three cysteines with alanines. The resulting constructs (C592A, C603A, and C608A) were stably expressed in HEK293 cells using the bicistronic vector pCIN4 (28), and total cell lysates were analyzed by Western blotting in the presence of increasing concentrations of DTT (Fig. 3). As in the experiment described in the previous paragraph, NEM was present both in the lysis buffer and in the SDS-polyacrylamide gel loading buffer. Fig. 3 shows that, for ABCG2-wt, the amount of monomer increased with the DTT concentration and that 10–50 mM was required for total reduction (Fig. 3D). Interestingly, the disulfide bridge-linked dimer was completely absent in the C603A mutant (Fig. 3B). In C592A and C608A, the disulfide-linked dimer was still present, although only scarcely in Cys-608 (Fig. 3, A and C). We observed also, however, a significant amount of monomer in the unreduced samples of C592A and C608A as compared with wt (Fig. 3, A and C).

Note that, in all Western blots for which we used the monoclonal BXP-21 antibody, it was observed that the intensity of the fully reduced monomer band is lower than that of the corresponding dimer band. It was observed even though the same amount of protein had been loaded in all of the lanes and the samples were uniformly blotted onto the membrane. We have no obvious explanation for this phenomenon, which also has been reported by others (20), except that it could relate to a difference in the reactivity of the antibody toward the monomeric as compared with the dimeric ABCG2.

Subsequent immunocytochemistry analysis of the single mutants was in agreement with the Western blot analysis and supported that the wt and C603A mutant were expressed almost exclusively in the plasma membrane (Fig. 3, B and D). In comparison, C592A and C608A exhibited less apparent plasma membrane staining and substantially more intracellular staining than in wt and C603A (Fig. 3, A and C).

To evaluate the influence of the cysteine mutations on transporter function, we performed a cell survival assay measuring the resistance to mitoxantrone. Fig. 4A shows the inhibition curves from a single experiment, whereas the histogram in Fig. 4B represents normalized LC50 values (mean ± S.D. of three separate experiments). ABCG2-wt showed a marked resistance to mitoxantrone compared with non-transfected HEK293 cells (Fig. 4, A and B). Only in C603A, the resistance...
to mitoxantrone was directly comparable with wt, whereas C592A and C608A showed significant decrease in resistance of ~50 and 70%, respectively (Fig. 4B). Because the mitoxantrone resistance is dependent on the expression of the transporter, we did a densitometry analysis of several Western blots to assess overall expression for comparison with the functional data. The analysis was performed on the 100 mM DTT samples, and the results are shown in Fig. 4C. The analysis showed that, although C603A tended to have a higher total expression, there were no significant changes in the expression of C592A and C608A. It is, however, important to correlate this with the immunostainings; i.e. both C592A and C608A displayed less apparent plasma membrane staining and increased intracellular staining, possibly accounting for the reduced activity (Fig. 4B).

Altogether, the data support a scenario in which Cys-603 forms a symmetrical intermolecular disulfide bridge that apparently is not crucial for proper targeting and function of the transport protein. The impaired targeting of C592A and C608A led us to hypothesize that these two residues could form a structurally important intramolecular disulfide bridge.

To further explore this hypothesis, we mutated two cysteines at a time, resulting in C592A/C603A, C592A/C608A, and C603A/C608A, each of which contained one remaining extracellular cysteine. Fig. 5 shows the reduction of the double cysteine mutants with increasing DTT concentrations. In C592A/C608A, which contained only Cys-603, we observed, in agreement with our hypothesis, efficient dimerization (Fig. 5D). The expression of C592A/C608A was also high and, in fact, increased as compared with the wt (Fig. 6C). In contrast, protein expression was very low in the construct containing only Cys-592 (C603A/C608A) (Figs. 5C and 6C) and slightly lower than wt for C592A/C603A, in which only Cys-608 is present (Figs. 5A and 6C). Notably, we also detected dimer formation in C603A/C608A and C592A/C603A. How-
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FIGURE 6. The presence of only Cys-603 retains function, whereas the presence of only Cys-592 or Cys-608 greatly decreases resistance to mitoxantrone. A, comparison of cell survival between empty HEK293 (○) and ABCG2-wt (●), C592A/C603A (▲), C592A/C608A (■), and C603A/C608A (□). Stable transfectants of HEK293 cells were seeded in 96-well plates and grown for 24 h. Mitoxantrone was added in increasing concentrations (0–6 μM final concentration) and incubated for 72 h at 37 °C. The cells were fixed, and cell survival was measured by the SRB method. Representative curves of the cytotoxicity assay are shown, where each point is measured in six individual wells. B, LC_{50} values were determined in Sigmaplot version 8.0 and represented normalized to wt. Normalized survival is shown as LC_{50}/LC_{50} (wt) for each construct. The data are means ± S.D.; n = 3. ***, significantly different from wt, p < 0.001. C, expression was assessed by densitometry analysis of Western blots of total lysates visualized by the BXP-21 antibody. The histogram shows the expression normalized to wt represented as the mean ± S.D.; n = 3.

However, these observations also lend support to our hypothesis; hence, a putative intramolecular disulfide bridge between Cys-592 and Cys-608 cannot be formed in these constructs (as well as in the single mutants of Cys-592 and Cys-608), leaving a cysteine free to form a non-native intermolecular disulfide bridge during protein folding and maturation. In contrast to the predicted native disulfide bridge involving Cys-603, such a disulfide bridge might be expected to interfere with targeting/function of the transporter.

This was further supported by immunocytochemistry and functional analysis. Staining of the transfected HEK293 cells showed that, although C592A/C608A (Fig. 5D) mostly resembled the membrane-localized expression pattern of wt (Fig. 5B), C592A/C603A displayed largely intracellular staining (Fig. 5A), and C603A/C608A was hardly visible (Fig. 5C). In further consistency with these observations, the cell survival assay revealed that Cys-603 alone displayed mitoxantrone resistance close (although not identical) to the wt (~70%) (Fig. 6, A and B), whereas both Cys-608 alone and Cys-592 alone displayed an almost identical low resistance of ~10 and 5% of the wt, respectively (Fig. 6, A and B). The histogram in Fig. 6B represents the same average values of three experiments as the one shown in Fig. 6A. The overall expression of the constructs based on densitometry analysis is shown in Fig. 6C. The analysis correlated generally well with the functional data. For both C603A/C608A and C592A/C603A, we observed a marked decrease in resistance to mitoxantrone and a concomitant decrease in expression (Fig. 6, B and C). For C592A/C608A, we observed, nonetheless, an increase in expression (Fig. 6C). Together with the slightly decreased function (Fig. 6B), this might suggest at least a partial functional impairment in this construct that contains Cys-603 as the only extracellular cysteine.

We also substituted all three extracellular cysteines in ABCG2 simultaneously (C592A/C603A/C608A). This inhibited completely the disulfide bridge-linked dimerization of the transporter (Fig. 7A) and decreased considerably both transporter function (Fig. 8, A and B) and protein expression (Figs. 7A and 8C). Specifically, we were almost unable to detect any expressed protein in the immunostainings (Fig. 7B).

To further explore the function of the hypothesized disulfide bridges, we performed efflux experiments on the mutants containing either both Cys-592 and Cys-608 (C603A), predicted to form an intramolecular disulfide bridge, or Cys-603 only (C592A/C608A), predicted to form an intermolecular disulfide bridge (Fig. 9). For mitoxantrone, we found the same pattern as in the cytotoxicity assay. C603A displayed efflux similar to wt, whereas efflux in C592A-C608A was slightly decreased (Fig. 9). The pattern was, however, different when analyzing another substrate for ABCG2, BODIPY-prazosin; i.e. we observed BODIPY-prazosin efflux similar to the wt in C603A, whereas in C592A-C608A we could not detect any evidence for BODIPY-prazosin efflux (Fig. 9).

To obtain additional biochemical evidence for the existence of the intra- and intermolecular disulfide bridges, we performed a surface biotinylation-labeling experiment using the membrane-impermeable sulphydryl reactive biotinylation reagent biotin-maleimide (32). The experiment was done with or without preincubation with the reducing agent TCEP (31). Without prior TCEP reduction, we observed no label-
antibody (5D3) (15) directed against an extracellular epitope of ABCG2.

Interestingly, the staining patterns were dissimilar with the two different antibodies. Of all mutants tested, only C603A showed clear staining with anti-ABCG2 (5D3) (Fig. 11); all other mutants showed no staining (Fig. 11). Because this is in clear contrast to our observations with the intracellular antibody, it is conceivable that mutation of either Cys-592 or Cys-608, and hence removal of the intramolecular disulfide bridge, might affect the structural integrity of the extracellular loop and, as a result, impair antibody binding.

**DISCUSSION**

In this study, we have explored the role of three predicted extracellular cysteines for the structure and function of the multidrug resistance half-transporter ABCG2. We and others have previously provided evidence that ABCG2 is a disulfide bridge-linked dimer (20, 24). ABCG2 migrates on SDS-PAGE corresponding to the size of a dimer under non-reducing conditions but as a monomer under reducing conditions. From the predicted topology of ABCG2, it is apparent that it contains three extracellular cysteines, Cys-592, -603, and -608, in the loop between TM5 and TM6. We sequentially mutated one, two, or all three of these cysteines and analyzed the influence of the mutations on transporter function and formation of DTT-reducible dimers. From these experiments, several lines of evidence supported that Cys-603 is responsible for ABCG2 being a disulfide bridge-linked dimer. First, mutation of this residue alone disrupted the apparent dimerization without affecting protein expression, as observed by Western blotting. This was not the case upon mutation of Cys-592 or Cys-608. Second, mutation of Cys-592 and -608 together preserved dimerization. Third, the presence of an extracellular disulfide bridge in the latter double mutant was also directly supported by our biotinylation experiments (Fig. 10). According to the predicted topology of ABCG2, there are no other cysteines in ABCG2 exposed to the extracellular environment, and thus, it is most likely that Cys-603, in two adjacent transporter molecules, forms a symmetrical disulfide bridge.

The putative Cys-603 disulfide bridge appeared not to be essential for transporter expression, targeting, and function. Given that previous evidence supports that ABCG2 (24), as well as other both prokaryotic and eukaryotic half-transporters are functional dimers (18, 33–35), it is therefore most likely that the intermolecular disulfide bridge involving Cys-603 is not a prerequisite for transporter dimerization in the native membrane. The unconstrained plasma membrane targeting of C603A also indicates that we have not disrupted dimerization in the native membrane, because evidence has been obtained in the homologous
ABCG5 and ABCG8 transporters supporting the finding that dimerization is critical for endoplasmic reticulum export (35, 36). Thus, mutation of Cys-603 is probably not disrupting non-covalent dimerization of ABCG2, and accordingly the mutant transporter is most likely held together as a dimeric complex in the native membrane by non-SDS-resistant, non-covalent interactions in the living cell. Of additional interest is the demonstration that a dimerization motif, GXXXG (25, 26), in TM1 of ABCG2 is involved in maintaining the structural integrity of the transporter, but it is clear that the motif is not the sole determinant of dimerization of ABCG2 (27). Finally, we must note that the transmembrane domains of ABC transporters show very low sequence identity, and it is therefore not unlikely that different interactions are involved in dimerization of different ABC half-transporters.

The present data are also consistent with the existence of an intramolecular disulfide bridge between Cys-592 and Cys-608. Individual mutation of each of these two residues results in decreased plasma membrane targeting and, as a likely consequence of this, decreased function according to the cytotoxicity assay. However, simultaneous mutation of both cysteines (C592A/C608A) restored plasma membrane targeting, and the expression even tended to be higher than that observed for the wt (Fig. 5). The most plausible explanation for these findings is the formation of an intramolecular disulfide bridge that is not essential for expression and targeting. In addition, biochemical evidence for the existence of this disulfide bridge was obtained from the biotinylation experiment showing biotinylation in C603A only with prior TCEP reduction (Fig. 10). It should be emphasized that Cys-592 and Cys-608 are very unlikely to form an intermolecular disulfide bridge in the wt protein, because mutation of Cys-603 alone (and thus with Cys-592 and Cys-608 still present) leads to disruption of the observed disulfide bridge-linked dimer. Cys-592 and Cys-608 might nonetheless form intermolecular disulfide bridges when they are “alone” as indicated from the results with the double mutations; i.e. both in C603A/C608A (where Cys-592 is alone) and in C592A/C603A (where Cys-608 is alone), we observed some disulfide bridge-linked dimers despite the fact that Cys-603 is mutated. In conjunction with the decreased plasma membrane expression and the consequent decrease in the function of these two
double mutants, we would argue that this observation provides further support for an intramolecular disulfide bridge between Cys-592 and Cys-608. In the absence of its native partner, the single cysteine left over forms an aberrant disulfide bridge with the same (or another) cysteine in the adjacent transporter molecule, leading to a structural perturbation that affects targeting/expression and thereby function of the transport protein.

In this context, it is also interesting to pay attention to the finding that, in the single mutants (C592A and C608A), we observed a significant amount of monomer in the unreduplicated samples (Fig. 3, A and C). The most conceivable explanation for the increased amount of monomer is the presence of an unpaired cysteine (Cys-592 or Cys-608). As for the double mutants, the remaining cysteine is capable of forming aberrant disulfide bridges; however, although in the double mutants the only possibility is an intermolecular aberrant disulfide bridge, an intramolecular disulfide bridge with the nearby Cys-603 is possible in the single mutants. This might inhibit formation of the Cys-603 intermolecular disulfide bridge, and consequently more monomer is observed on the Western blots. An alternative scenario could have been the formation of an aberrant intermolecular disulfide bridge by the unpaired cysteine, but this is not what we see possible, because the intramolecular bridge is more likely to be formed.

Mutating all three extracellular cysteines in ABCG2 (C592A/C603A/C608A) at the same time had detrimental effects on the transporter. The expression was reduced substantially and hence resistance to mitoxantrone was greatly decreased, and no evidence for disulfide bridge-linked dimerization was observed. This supports the conclusion that, although we can disrupt the putative intermolecular symmetrical disulfide bridge involving Cys-603 (C603A mutant) and although we can remove the putative intramolecular disulfide bridge between Cys-592 and Cys-608 (C592A/C608A mutant) without any major impact on expression of the transporter, it is not possible to remove both of them simultaneously. Thus, together the two disulfide bridges appear to be of fundamental importance for the structural integrity of the ABCG2 transporter.

Interestingly, our data also suggest that the intramolecular disulfide bridge between Cys-592 and Cys-608 could play a role in substrate specificity and transport. In C592A/C608A, we see no efflux of the substrate BODIPY-prazosin, although efflux of mitoxantrone is preserved. It is reasonable to reconcile this with the finding that the epitope for the extracellular antibody anti-ABCG2 (5D3) (15) was disrupted by mutation of Cys-592 and Cys-608 alone or together (Fig. 11). In combination, the data suggest that removal of the disulfide bridge alters the structure of the loop, and as a consequence, the substrate specificity of the transporter changes. It is also reasonable to reconcile the observation with a recent study by Ozvegy-Laczka et al. (37) showing that reactivity of the anti-ABCG2 antibody 5D3 was altered under different circumstances, including the addition of nucleotides and inhibitors of ABCG2. Thus, it was proposed to be a sensor for conformational changes within ABCG2 during ATP hydrolysis. Because the current data provide evidence that the epitope for 5D3 must be present in the extracellular loop between TM5 and TM6, it is tempting to suggest that binding of nucleotide to the transporter leads to conformational changes in the extracellular loop that are critical for substrate transport and specificity. The impact of this observation awaits further investigation.

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