Single-nucleotide polymorphisms in a short basic motif in the ABC transporter ABCG2 disable its trafficking out of endoplasmic reticulum and reduce cell resistance to anticancer drugs

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ABCG2 is a member of the ATP-binding cassette (ABC)4 transporter superfamily (1) with multiple substrates including physiological metabolites and chemotherapeutics (2, 3). Loss-of-function mutations in ABCG2 cause genetic diseases including gout (4) and porphyria (5). ABCG2 overexpression, however, contributes to multidrug resistance in cancer chemotherapy (6). ABCG2 has also been associated with side populations of hematopoietic stem cells (7) and possibly responsible for the drug-resistance property of cancer stem cells (8).

ABCG2 is considered a half transporter with one nucleotide-binding domain (NBD) and one transmembrane domain (TMD) consisting of six transmembrane segments (Fig. 1A), whereas full transporters such as ABCB1 has two NBDs and two TMDs (9). Thus, ABCG2 has been thought to function as a homodimer, supported by recent cryo-EM structure (10), although prior biochemical and EM studies showed existence of higher orders of complexes (11–13) with TMD responsible for oligomerization (14, 15).

The NBD of ABC transporters consists of three motifs including a Walker A motif, a Walker B motif, and a signature motif or C motif with a consensus sequence of LSGGQ (16). The C motif in ABCG2 has a conserved sequence of 186VSGGE (17) with known nonsynonymous single-nucleotide polymorphisms (SNPs) and potential phosphorylation sites (Fig. 1A, inset).

In this study, we investigated the potential role of the positive charges and the putative phosphorylation sites downstream of the C motif in ABCG2 biogenesis and function. We found that the putative phosphorylation sites may not be phosphorylated.
However, the positive charges are essential for the successful trafficking and maturation of ABCG2. The SNPs that reduce these positive charges are unable to mature and accumulate in the ER but induce ER stress and degradation of the mutant ABCG2 via ER stress–associated degradation (ERAD). Mutant ABCG2 with reduced positive charges can still bind to but is unable to hydrolyze ATP. These findings suggest that the SNPs that reduce positive charges may confer better prognosis of cancer patients and may also cause genetic diseases.

Results

The positive charges downstream of the C motif regulate ABCG2 biogenesis

Sequence analyses using an online tool (https://scansite.mit.edu/4.0/) (50) showed two potential AKT phosphorylation sites, Thr\textsuperscript{194} and Ser\textsuperscript{195}, downstream of the C motif in ABCG2 (Fig. 1A). To investigate whether these sites are important, we mutated Thr\textsuperscript{194} and Ser\textsuperscript{195} to create nonphosphorylatable (T194A, S195A, and T194A/S195A [AA]) and phosphorylation mimic (T194D, S195D, and T194D/S195D [DD]) mutants (Table 1). These mutants were then expressed as Venus-tagged fusion proteins for convenience of subcellular localization study in HEK293 cells.

As shown in Fig. 1 (B and C), although T194A, S195A, and AA mutants had expression patterns similar to that of WT ABCG2 with a major product at ~110 kDa, the expression of T194D, S195D, and DD mutants were dramatically reduced with a major product at ~90 kDa. Semiquantitative (Fig. 1D) and quantitative (Fig. 1E) RT-PCR analyses revealed similar mRNA levels for all mutant and WT ABCG2. WT and mutant GST-tagged ABCG2 had expression profiles similar to that of Venus-tagged ABCG2 (Fig. 1F). Thus, the altered expression profile and level of the T194D, S195D, and DD mutant ABCG2 are unlikely because of different transfection efficiency, difference in transcription, or use of Venus tag.

We next determined whether the 110-kDa Venus-tagged ABCG2 represents fully glycosylated mature ABCG2, and the 90-kDa protein is the immature one with core glycosylation by dissecting glycosylation status using endoglycosidase PNGase F and Endo H. Although PNGase F can remove both immature and mature N-linked glycoproteins (22), Endo H cleaves only high mannose but cannot cleave mature polycarbohydrates (23). As shown in Fig. 2A, both the 110-kDa and 90-kDa proteins of WT and mutant ABCG2 were sensitive to and were reduced to 85 kDa following PNGase F treatment. However, the 110-kDa protein was resistant, whereas the 90-kDa protein was sensitive to Endo H treatment (Fig. 2B). Thus, the 110-kDa protein likely represents the mature fully glycosylated, whereas the 90-kDa protein represents immature core-glycosylated ABCG2 and that ABCG2 biogenesis (expression, trafficking, and maturation) was drastically reduced by mutation of Thr\textsuperscript{194} and Ser\textsuperscript{195} to Asp.

Table 1

| Construct names | C motif and mutations | Charge distributions |
|-----------------|-----------------------|----------------------|
| Wildtype        | Wildtype              | ++                   |
| T194A           | Wildtype              | ++                   |
| T194D           | Wildtype              | ++                   |
| S195A           | Wildtype              | ++                   |
| S195D           | Wildtype              | ++                   |
| AA              | Wildtype              | ++                   |
| DD              | Wildtype              | ++                   |
| KKK             | Wildtype              | ++                   |
| AAA             | Wildtype              | ++                   |
| EEE             | Wildtype              | ++                   |
| R191S           | Wildtype              | ++                   |
| K192E           | Wildtype              | ++                   |
| T194I           | Wildtype              | ++                   |

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To confirm the above findings, we treated these cells with brefeldin A, which inhibits trafficking of nascent membrane proteins from ER to Golgi (24). As shown in Fig. 2C, 100 μM brefeldin A inhibited production of the 110-kDa mature but increased that of the 90-kDa immature WT ABCG2. However, it had no effect on the expression profile of the DD mutant ABCG2. Consistent with above findings, confocal imaging of venus-tagged ABCG2 showed that most of the WT and AA mutant, whereas little of the DD mutant ABCG2 was localized on plasma membranes (Fig. 2D).

The above findings are peculiar because mutation of Thr<sup>194</sup> and Ser<sup>195</sup> to Ala to eliminate potential phosphorylation of these residues did not affect the biogenesis while mutating them into Asp to mimic phosphorylation dramatically reduced the ability of ABCG2 to express and mature. However, it is possible that phosphorylation of these residues affects ABCG2 biogenesis, and these residues may not be phosphorylated in HEK293 cells. To test this possibility, we transfected and overexpressed AKT in the cells expressing ABCG2 and examined its effect on ABCG2 biogenesis. Overexpressing AKT did not reduce the expression or glycosylation of WT or DD mutant ABCG2 (data not shown). Thus, Thr<sup>194</sup> and Ser<sup>195</sup> may not be phosphorylated by AKT that leads to reduced ABCG2 biogenesis.

Sequence analysis showed that there are three positively charged residues: RKR<sup>193</sup> preceding Thr<sup>194</sup> and Ser<sup>195</sup>. Thus, it is possible that mutating Thr<sup>194</sup> and Ser<sup>195</sup> into Asp may have changed the charge environment downstream of the C motif, which may affect ABCG2 biogenesis. To test this possibility, we mutated RKR<sup>193</sup> to KKK, AAA, or EEE (Table 1) and performed

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**Table 1**

| Residue | WT | T<sup>194</sup>A | T<sup>194</sup>D | W<sup>195</sup>A | W<sup>195</sup>D |
|---------|----|-----------------|-----------------|---------------|---------------|
| Thr     | 85 | 85              | 85              | 85            | 85            |
| Ser     | 55 | 55              | 55              | 55            | 55            |
| Arg     | 43 | 43              | 43              | 43            | 43            |

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**Figure 2. Glycosylation status and subcellular localization of WT and mutant ABCG2.** A and B, glycosylation status. WT and mutant ABCG2 in HEK293 cell lysates were subjected to digestion by endoglycosidase PNGase F (A) or Endo H (B) followed by Western blotting analysis. C, retrograde transport of ABCG2. HEK293 cells expressing WT or mutant ABCG2 were treated with brefeldin A (BA) at different concentrations as indicated for 24 h followed by Western blotting analysis of ABCG2. D, subcellular localization of WT and mutant ABCG2 as determined using confocal imaging of the Venus tag of ABCG2. Actin was used as a loading control for Western blotting. DAPI and DiD were used to counterstain nuclei and plasma membranes in imaging analysis. ** and * indicate mature fully glycosylated and core-glycosylated ABCG2, respectively. The arrow indicates unglycosylated ABCG2.
the same experiments as described above. As shown in Figs. 1 (B–F) and 2D, except the KKK mutation that did not change the positive charges, both AAA and EEE mutants are defective in biogenesis, similar to the DD mutant. Thus, the positive charges downstream of the C motif may play an important role in regulating ABCG2 biogenesis.

**Nonsynonymous SNPs of the positively charged residues reduce ABCG2 biogenesis**

To investigate whether the above findings have any physiological relevance, we searched the SNP database (https://www.ncbi.nlm.nih.gov/projects/SNP/) and identified three nonsynonymous SNPs, R191S, K192E, and T194I downstream of the human ABCG2 C motif. To determine whether these SNPs may affect ABCG2 biogenesis, we recreated these mutations and performed the same experiments as described above. As shown in Figs. 1 (B–F) and 2 (A–D), T194I mutation, which does not change the charges, had no effect, whereas the other mutations (R191S and K192E) that reduce positive charges dramatically reduced ABCG2 biogenesis. These findings not only confirm that the positive charges downstream of the C motif regulate ABCG2 biogenesis but also suggest that these positively charged residues may be physiologically relevant.

**Reducing positive charges induces ABCG2 degradation in proteosome**

To understand how decreasing positive charges downstream of the C motif reduces ABCG2 expression, we first performed a cycloheximide-chasing experiment to determine the half-life of WT versus mutant ABCG2 as described previously (25). As shown in Fig. 3 (A and B), the half-life of DD, R191S, and K192E mutant ABCG2 is estimated to be 0.6–4 h, whereas the WT ABCG2 is much more stable with a half-life of >10 h. Thus, the loss of positive charges downstream of the C motif likely accelerates ABCG2 degradation.

Next, we used MG132 and bortezomib, the proteasome inhibitors, and bafilomycin A1 (BFA1), an inhibitor of protein degradation in lysosomes, to elucidate the degradation pathways of the WT and mutant ABCG2. As shown in Fig. 3C, whereas BFA1 increased the expression level of WT ABCG2, the DD, R191S, and K192E mutant ABCG2 were mostly rescued by MG132 and bortezomib. These findings suggest that the WT ABCG2 is mostly degraded in lysosome, consistent with previous findings (26, 27), while the mutant ABCG2 with reduced positive charges are mainly degraded in proteosomes.

**ERAD of mutant ABCG2 with reduced positive charges**

Because loss of positive charges downstream of the C motif inhibits ABCG2 trafficking from ER to plasma membranes and induces ABCG2 degradation in proteasomes as shown above, we hypothesized that these mutations might increase accumulation of the mutant ABCG2 in ER by inhibiting its trafficking, resulting in ER stress and degradation of these mutant ABCG2 via ERAD pathway. To test this hypothesis, we used mitoxantrone and doxorubicin to induce ER stress as previously described (28–31) and determined the expression level of DD mutant as a representative along with the WT ABCG2. As shown in Fig. 4A, both mitoxantrone and doxorubicin induced ER stress as indicated by up-regulation of the ER stress marker CHOP. The expression of WT ABCG2 was also slightly increased by mitoxantrone and doxorubicin, consistent with previous findings that these ABCG2 substrates function as a chemical chaperone for ABCG2 (4, 32). However, the expression of the DD mutant was further reduced following mitoxantrone and doxorubicin treatment, possibly because of increased ER stress.

To determine whether the further reduction in the expression of the DD mutant following mitoxantrone or doxorubicin treatment was due to increased degradation in proteasomes, we...
Polymorphisms and biogenesis of ABCG2 in drug resistance

Figure 4. ER stress, expression, and degradation of ABCG2. A and C, effect of ER stress inducer (A) and inhibitor (C) on WT and the DD mutant ABCG2 expression. HEK293 cells expressing WT and the DD mutant ABCG2 were treated with ER stress inducer mitoxantrone or doxorubicin (A) or inhibitor 4-PBA (C) at different concentrations as indicated for 24 h followed by isolation of cell lysates for Western blotting analysis of ABCG2 and ER stress marker CHOP and calnexin. B, reversal of ER stress inducer ABCG2 reduction. HEK293 cells expressing WT or the DD mutant ABCG2 were treated with 5 μM mitoxantrone or 5 μM doxorubicin together with 0.5 μM MG-132 or 0.5 μM BFA1 for 24 h followed by isolation of cell lysates for Western blotting analysis of ABCG2. Actin was a loading control.

performed rescue experiment using MG132. As shown in Fig. 4B, mitoxantrone- or doxorubicin-induced reduction in DD mutant expression was fully reversed by MG132. However, BFA1 rescued little in production of the DD mutant ABCG2 in proteasomes.

To further understand the mechanism of ERAD of mutant ABCG2, we tested whether inhibiting ER stress could rescue mutant ABCG2 from degradation. 4-PBA is a short-chain fatty acid that has been used as an ER stress inhibitor (33). 4-PBA treatment decreased the expression of ER stress markers (GRP78, ATF6, ATF4, and CHOP) (34) and rescued the plasma membrane targeting of mutant ABC proteins including ABCA1 (35), ABCB11 (36), ABCC1 (37), ABCC6 (38), and ABCC7 (39). As shown in Fig. 4C, 4-PBA treatment increased the expression of the DD mutant in a dose-dependent manner. It had no effect on the expression of WT ABCG2 as expected. Interestingly, the expression of the stress markers including calnexin and CHOP also increased by 4-PBA in cells expressing the DD mutant but not in cells expressing the WT ABCG2. The increase in ER stress marker may be due to 4-PBA-induced accumulation of mutant ABCG2 in the ER that further increases ER stress. Based on these findings, we conclude that the mutant ABCG2 with reduced positive charges downstream of the C motif likely fails to traffic out of and accumulates in the ER, which leads to degradation of these proteins via ERAD.

The functional NBD is essential for successful trafficking and maturation of ABCG2

To further understand the mechanism of ERAD of mutant ABCG2 with reduced positive charges downstream of the C motif, we next tested the effect of these mutations on the activity of ABCG2 in ATP binding and hydrolysis. We hypothesized that the loss of positive charges downstream of the C motif might eliminate the binding of negatively charged ATP and ATPase activity. To test this hypothesis, we first performed a pulldown assay of WT and mutant ABCG2 using immobilized ATP. As shown in Fig. 5A, both mature and immature WT and mutant ABCG2 were effectively pulled down by immobilized ATP, suggesting that they all can bind to ATP. We also performed the same experiment by pretreating cell lysate with vanadate to induce vanadate trapping and to dissect active from inactive ATPase because only the active one can trap vanadate. As shown in Fig. 5B, the WT and the KKK mutant ABCG2 could not be pulled down after vanadate pretreatment, whereas the other mutant ABCG2 with reduced positive charges could still be pulled down by the immobilized ATP. These findings suggest that the WT and the KKK mutant ABCG2 has active ATPase activity with the ability to bind to and hydrolyze ATP, whereas the other mutant ABCG2 with reduced positive charges are able to bind to ATP but may have lost ATPase activity.

To confirm the above findings, we next tested the drug-stimulated and vanadate-sensitive ATPase activity of the cells expressing WT and three mutant ABCG2 with reduced positive charges downstream of the C motif. As shown in Fig. 5C and D, only cells expressing the WT ABCG2 showed significant and strong drug-stimulated vanadate-sensitive ATPase activity. Interestingly, cells expressing the R191S mutant with minimal mature ABCG2 also has minimal but significant drug-stimulated vanadate-sensitive ATPase activity, whereas cells expressing the other mutants do not have any. These findings not only confirm that the mutant ABCG2 with reduced positive charges loses its ATPase activity but also suggest that the ATPase activity may be important for ABCG2 trafficking out of ER.

To test this possibility, we created Venus- or FLAG-tagged TMD constructs of ABCG2 by deleting the NBD followed by analysis of its maturation and subcellular localization. As shown in Fig. 5E, the TMD of ABCG2 are sensitive to both PNGase F and Endo H treatments, suggesting that it had no mature complex glycosylation and, thus, unlikely matured through Golgi onto plasma membranes. Indeed, fluorescence imaging analysis showed that Venus-TMD are localized intracellularly compared with the WT full-length Venus–ABCG2, which are primarily on plasma membranes (Fig. 5F). Thus, the functional NBD domain is necessary for trafficking and maturation of ABCG2.

Dimerization of the mutant ABCG2 with reduced positive charges

As discussed above, ABCG2 is thought to exist as a homodimer. It is also thought that dimerization of ABCG subfamily members including ABCG2 takes place in the...
ER and is required for surface expression (40). To determine whether mutant ABCG2 with reduced positive charges lost its dimerization activity, leading to defective trafficking and maturation of ABCG2, we co-expressed WT and the DD mutant ABCG2 with GST or Venus tag followed by pulldown assay by taking advantage of the GST tag and Western blotting analysis of Venus-tagged ABCG2. As shown in Fig. 6, the GST-tagged DD mutant was able to interact with Venus-tagged WT and mutant ABCG2 and vice versa. Thus, the mutation unlikely disrupts dimerization that leads to defective trafficking and maturation of ABCG2.

**The mutant ABCG2 with reduced positive charges are inactive in drug transport and resistance**

Because the mutant ABCG2 with reduced positive charges downstream of the C motif in NBD are unable to hydrolyze ATP and cannot successfully traffic onto plasma membranes, it is conceivable that this mutant ABCG2 is unlikely functional in drug transport and resistance. To ensure that this is the case, we first performed mitoxantrone accumulation study using live cells expressing WT and mutant ABCG2 and FACS analysis. As shown in Fig. 7 (A and B), mitoxantrone accumulation normalized to the level of ABCG2 varies among mutant ABCG2. The mutants that have mature ABCG2 were able to reduce mitoxantrone accumulation whereas the mutants that have no mature ABCG2 lost their activity in reducing mitoxantrone accumulation. There is a strong negative correlation with a coefficient of −0.8 (Fig. 7D) between the level of mitoxantrone accumulation (Fig. 7B) and the level of mature fully glycosylated ABCG2 (Fig. 7C). Survival analysis of cells expressing the WT and the DD mutant ABCG2 also showed that the DD mutant was unable to confer resistance to mitoxantrone with a resistance factor of 1.3, whereas the WT ABCG2 has a resistance factor of 18.2 (data not shown). Thus, the mutant ABCG2 that cannot mature is functionally dead in drug transport and drug resistance.

**Discussion**

In this study, we found that the positive charges immediately downstream of the C motif plays an important role in ABCG2 biogenesis. Lessening this positivity disables the trafficking of and accumulates nascent ABCG2 in the ER, which causes ER

![Figure 5. ATP binding and hydrolysis activity of WT and mutant ABCG2.](image)

**Figure 5. ATP binding and hydrolysis activity of WT and mutant ABCG2.** A and B, pulldown assay. Lysate from cells expressing WT or mutant ABCG2 were pretreated without (A) or with (B) vanadate followed by pulldown assay using immobilized ATP and Western blotting analysis of pulldown materials. Note that the same input sample was used for the pulldown experiment of WT, KKK, AAA, and EEE in the absence (A) and presence (B) of vanadate. C and D, drug-stimulated vanadate-sensitive ATPase activity assay. Crude membranes were isolated from HEK293 cells expressing WT and mutant ABCG2 and subjected to Western blotting analysis (C) and ATPase activity assay (D). Na₃ATPase was used as a loading control. E, glycosylation status of the TMD of human ABCG2. Lysates from HEK293 cells expressing Venus- or FLAG-tagged TMD of ABCG2 were subjected to digestion by PNGase F or Endo H followed by Western blotting analysis of TMD and actin loading control. F, subcellular localization of Venus-TMD as determined using confocal imaging of the Venus tag. Full-length Venus–ABCG2 was used a control. The nuclei were counterstained using DAPI. ** and * indicate mature fully glycosylated and core-glycosylated ABCG2, respectively. The arrow indicates unglycosylated ABCG2.

![Figure 6. Effect of mutations on ABCG2-ABCG2 interaction.](image)
stress and ABCG2 degradation in proteasomes via ERAD (Fig. 8A). We also found existence of SNPs, which reduce these positive charges and effectively reduce ABCG2 expression and maturation.

Although the data suggest that the positive charges downstream of the C motif are critical for the trafficking and maturation of ABCG2, the potential impact of using HEK293 cells as a host and the overexpression system on ABCG2 trafficking is unknown. However, HEK293 cells have been widely used to express and study ABC transporters because of a lack of detectable expression level of endogenous ABC proteins. Compared with the high level of ectopic ABCG2, the undetectable level of endogenous ABCG2 unlikely causes major changes in the trafficking of the ectopic ABCG2. Furthermore, the trafficking of the overexpressed WT ABCG2 is normal in HEK293 cells, helping eliminate the concern in using overexpression in HEK293 cells.

The positively charged residues immediately downstream of the C motif, RKR, have previously been reported as a RKR motif that has an important role in ER retention of ATP-sensitive K⁺ channel protein Kir6.1/2 and SUR1 (21). Deleting or mutating the RKR motif enables Kir6.1/2 and SUR1 proteins, expressed alone, to traffic onto plasma membranes. However, mutating the RKR motif in ABCG2 disables the protein to mature and traffic onto plasma membranes. Thus, the RKR motif in ABCG2 likely have different functions from that in Kir6.1/2 and SUR1. Because mutating further downstream residues and adding negative charges also disabled ABCG2 trafficking and maturation, we believe that it is not the RKR motif per se but rather the overall positive charges downstream of the C motif that are important for ABCG2 biogenesis. Consistent with this argument is the finding that mutating RKR to KKK to maintain positive charges had no effect on ABCG2 biogenesis.

Because the degradation of mutant ABCG2 may occur in the proteasome, it is possible that the RKR motif constitutes a potential ubiquitination site and that mutating the RKR motif affects ubiquitination of ABCG2 at the RKR motif, resulting in accelerated degradation. Analysis of the ABCG2 sequence showed that ABCG2 has several potential ubiquitination sites at Lys358, Lys357, Lys323, Lys177, Lys172, and Gln166. However, Lys192 in the RKR motif is not predicted to be a potential ubiquitination site. Furthermore, mutating Lys192 to Glu192 in the K192E mutant resulted in a loss of expression, not increased expression of ABCG2, inconsistent with potential elimination of ubiquitination of ABCG2 at Lys192. Thus, the effect of these mutations on ABCG2 maturation and degradation is unlikely due to their effects on potential ubiquitination of Lys192.

Analysis of all 48 ABC proteins showed that the positive charges downstream of the C motif are conserved among most members of human ABC proteins (Fig. 8B). However, the ER-resident proteins ABCB2 (TAP1) and ABCB3 (TAP2) lack these positive charges. Another ABC protein that lacks these positive charges is ABCD4, which has also been shown to localize in the ER (41). ABCE1 is an example of ABC proteins that lack the TMD and, thus, does not need the traffic from ER to plasma membranes. Interestingly, ABCE1 also lacks the overall positive charges downstream of the C motif. These observations are interesting and consistent with the potential role of these positive charges in programing trafficking of ABC proteins out of ER to mature onto plasma membranes.

As discussed earlier, the C motif is a signature sequence for ABC transporters and potentially important for the ATPase activity of ABCG2. Thus, mutating the positive residues immediately downstream of this motif may affect ATP binding and hydrolysis. Interestingly, reducing the positive charges did not affect the ATP-binding activity, although it reduced the ATPase activity. This finding is peculiar and suggests that the mutation may not have induced major conformational change of the NBD. Currently, it remains unclear whether the loss of ATPase activity plays any role in ABCG2 trafficking and matu-
Nevertheless, it is tempting to speculate that ATP hydrolysis catalyzed by the ATPase may help propel the trafficking of nascent ABCG2 from ER through Golgi onto plasma membranes. However, previous studies of ABCB1 with mutation of R538M immediately downstream of the first C motif had no effect on ABCB1 expression and trafficking, although it reduced about 50% of ATPase activity of the protein (19). Mutations of the C motif in ABCC1 (G771D and G1433D) that created negative charges appear to affect ATPase activity but had little effect on expression of ABCC1 (20). Although these studies were performed in insect Sf9 cells, which may be different from mammalian cells in regulating membrane protein trafficking, these observations argue against the speculation that the functional ATPase may be required for biogenesis of ABC transporters.

Although ER accumulation of mutant ABCG2 may cause ER stress, we were unable to detect up-regulation of the ER stress marker CHOP and calnexin following expression of the mutant ABCG2 proteins. We believe that quick degradation of the mutant ABCG2 effectively releases the ER stress. This speculation was supported by the finding that inhibiting ER stress with 4-PBA reduced degradation of mutant ABCG2, which increased the expression of CHOP and calnexin. It is also noteworthy that mitoxantrone and doxorubicin are known ER stress inducers as well as substrates and chemical/pharmacological chaperones of ABCG2 (4, 32). The finding that mitoxantrone and doxorubicin further reduced the expression of the DD mutant ABCG2 via proteasome suggests that these agents were unable to bind to the DD mutant ABCG2 to perform its chaperone function. Although it is unlikely that the C motif and positively charged residues in NBD are involved in binding these drug substrates, the lack of functional ATPase in the DD mutant may be unable to support the chaperone function of mitoxantrone and doxorubicin. Thus, the ER stress-inducing activity of these drugs prevails, leading to accelerated ERAD of the DD mutant ABCG2.

The finding that the two SNPs (R191S and K192E) in the RKR motif that reduce positive charges, as well as ABCG2 expression and trafficking, has very important physiological implications. Individuals with these SNPs may demonstrate lowered transport of substances that are substrates of ABCG2, resulting in increased risks of diseases such as gout, hypertension, diabetes, nephropathy, and pleomorphism (42). However, cancer patients with these SNPs, including R191S and K192E, will likely have a better response to traditional and targeted chemotherapy with drugs that are ABCG2 substrates. Further studies of clinical samples and patient outcomes will help test these possibilities.
Polymorphisms and biogenesis of ABCG2 in drug resistance

Experimental procedures

Materials

PNGase F, Endo H, Eco53KI, Swal, T4 DNA ligase, alkaline phosphatase, and Na2VO4 were from New England BioLabs. Brefeldin A, cycloheximide, MG132, bortezomib, bafilomycin A1, 4-PBA, mitoxantrone, doxorubicin, camptothecin, ATP-agarose 4B, and the ATPase activity assay kit were from Sigma–Aldrich. Lipofectamine 2000, DAPI, protein assay kit, DNA and RNA extraction kits, the high-capacity cDNA reverse transcription kit, GatewayTM LR ClonaseTM II enzyme mix, Cddb and Stbl3 competent Escherichia coli were from Thermo Fisher Scientific. GoTaq Flexi DNA and PfuTurbo DNA polymerases are from Promega and Agilent, respectively. Antibodies against Na+/K-ATPase (3010), β-actin (3700), and horseradish peroxidase–conjugated anti-rabbit (7074), and antiamouse (7076) IgG were from Cell Signaling Technology. Antibodies against ABCG2 (MAB4155), GFP (sc-390394), and GST (27457701) were from Millipore, Santa Cruz Biotechnology, and GE Healthcare, respectively. pcDNA3.1-3xFLAG-V5-ccdB and pDEST27-GST were from Addgene. Biotechnology, and GE Healthcare, respectively. pcDNA3.1-3xFLAG-V5-ccdB and pDEST27-GST were from Addgene.

Construct engineering and site-directed mutagenesis

The new Gateway destination expression vector pCDH-Neo-Venus/dest was engineered by releasing the Venus-ccdB fragment from pSCM167 (43) using Eco53KI followed by insertion into pCDH-CMV-MCS-SV40-Neo linearized using SwaI. This high-copy eukaryotic expression vector has a cytomegalovirus promoter, Venus tag, antibiotics neomycin (Neo), and the Gateway destination recombination sites attR1 and attR2. Cloning of ABCG2 into pCDH-Neo-Venus/dest was conducted using pDONR221-ABCG2 as the entry vector and the Gateway LR Clonase system. To engineer the GST–ABCG2 fusion protein, pDEST27-GST was used as the destination vector.

Site-directed mutagenesis was conducted as we previously described (44) using pDONR221-ABCG2 as a template and specific pairs of primers for each mutation (Table S1). All mutations were validated using double strand DNA sequencing by GenScript.

Cell culture, transfection, and imaging

HEK293 cells from ATCC, which has been authenticated August 10, 2018, were propagated at 37 °C with 5% CO2 in high-glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Transient transfection were performed as we previously described (44). Briefly, ~1 × 106 HEK293 cells/well were plated on 6-well plates and cultured for 24 h before transfection of ABCG2 CDNA using LipofectamineTM 2000. The cells were harvested 48 h after transfection for different assays or subjected to selection using 1.2 μg/ml G418 for 10 days to generate pooled stable clones.

HEK293 cells expressing Venus–ABCG2 on cover glass were fixed for 10 min in 4% paraformaldehyde at room temperature, washed, and counterstained with DiD (Meilunbio, MB6190, 2 μM/ml) for 30 min at 37 °C and then with DAPI for 10 min at room temperature. The cells were rinsed three times with PBS, mounted, and then analyzed using a confocal microscope (Olympus 2).

RT-PCR and qRT-PCR

RT-PCR was performed as we previously described (27, 45, 46). Briefly, total RNAs were isolated from HEK293 cells expressing ABCG2 using RNA extraction kit, and 1 μg of total RNA was subjected to reverse transcription using a high-capacity cDNA reverse transcription kit followed by PCR using GoTaq Flexi DNA polymerase and specific primers (Table S2). The final RT-PCR products were analyzed by electrophoresis.

qRT-PCR was performed using qPCR master mix (Roche, LightCycler® 480 SYBR Green I Master) and specific primers (Table S3). 2∧ΔΔCT was used to analyze the relative expression level.

Cell lysate preparation, Western blotting, and ATP-binding assay

At 48 h following transfection, the cells were harvested, washed with PBS, and lysed in radioimmune precipitation assay buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 0.03% aprotinin, 1 mM sodium orthovanadate) at 4 °C for 30 min followed by centrifugation at 14,000 g for 10 min. The supernatants were collected and stored at ~80 °C. For ATP-binding assay, cells were lysed in TBS (100 mM Tris, pH 8.0, 150 mM NaCl) containing 1 mM PMSF and 1 mM DTT.

Western blotting analysis was performed as previously described (11, 14). Briefly, 10–50 μg of proteins was separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% BSA and incubated with primary antibodies followed by incubation with horseradish peroxidase–conjugated secondary antibodies. The signals were captured using X-ray films.

For ATP-binding assay, 200 μg of cell lysate was mixed with 50 μl of ATP-agarose pre-equilibrated in TBS and incubated in the presence of 1 mM PMSF and 1 mM DTT at 4 °C for 12 h with continuous mixing. The beads were washed three times with TBS, and the ATP-bound materials were separated by SDS-PAGE followed by Western blotting analysis.

Crude membrane isolation and ATPase activity assay

Crude membranes were prepared as previously described (14, 27, 47). Briefly, 48 h post-transfection, the cells were harvested and lysed in hypotonic lysis buffer (10 mM KCl, 1.5 mM MgCl2, 10 mM Tris, pH 7.4, 2 mM PMSF) on ice for 40 min followed by homogenization using a Dounce homogenizer and centrifugation at 4000 × g for 10 min. The supernatant was collected and centrifuged again at 100,000 × g for 90 min at 4 °C. The pellet was resuspended in STBS (5 mM Tris, pH 7.4, 50 mM NaCl, 8.6% sucrose) for ATPase activity assay.

The ATPase activity assay was performed as previously described (48) using a kit as directed by the manufacturer. Briefly, the freshly isolated membranes were incubated with or without 0.3 mM vanadate at 37 °C for 10 min and then incubated with 10 μM camptothecin or DMSO control at 37 °C for 3 min. Following addition of 4 mM ATP and incubation at 37 °C for 30 min, the reaction was stopped for determination of Pi released by analyzing A620 nm using a microplate reader (BioTek Instru-
Polymorphisms and biogenesis of ABCG2 in drug resistance

HEK293 cells expressing ABCG2 were cultured on 6-well plates at 2.5 × 10⁵ cells/well and incubated with 20 µM mitoxantrone for 30 min and then in fresh medium without mitoxantrone for 1 h at 37 °C. The cells were harvested and analyzed using flow cytometry with excitation at 635 nm and emission at 661 nm [49].

Statistical analysis

All statistical analyses were performed using Prism 6.0 software (GraphPad Software, La Jolla, CA) and presented as the means ± S.E. The means were compared by ordinary one-way analysis of variance and Tukey’s multiple comparisons test.

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20232 J. Biol. Chem. (2019) 294(52) 20222–20232