Intramolecular Disulfide Bond Is a Critical Check Point Determining Degradative Fates of ATP-binding Cassette (ABC) Transporter ABCG2 Protein*

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Human ABCG2 belongs to the ATP-binding cassette (ABC) transporter family and plays an important role in various biological reactions, such as xenobiotic elimination and homeostasis of protoporphyrin. We previously reported that ABCG2 exists in the plasma membrane as a homodimer bound via a disulfide bond at Cys-603. In the present study, we examined the importance of an intramolecular disulfide bond for stability of the ABCG2 protein. Substitution of either Cys-592 or Cys-608 located in the extracellular loop to glycine resulted in a significant decrease in protein levels of ABCG2 when expressed in Flp-In-293 cells. Interestingly, the protein levels of those ABCG2 variants were remarkably enhanced by treatment with the proteasome inhibitor MG132. On the other hand, the protein level of ABCG2 WT increased more than 4-fold when cells were treated with bafilomycin A1, which inhibits lysosomal degradation, whereas the C592G variant was little affected by the same treatment. These results strongly suggest that two distinct pathways exist for protein degradation of ABCG2 WT and mutants lacking the intramolecular disulfide bond. The human ABCG2 exists in the plasma membrane as a homodimer (7–9). We previously demonstrated that human ABCG2 existed in the plasma membrane as a homodimer bound through disulfide-bonded cysteine residues. Treatment with mercaptoethanol reduced the apparent molecular weight of ABCG2 from 140,000 to 70,000 (9). Based on the cDNA sequence, a total of 12 cysteine residues exist in the ABCG2 protein (see Fig. 1). Although the cytosolic space is reductive due to the protection by antioxidants such as glutathione, the extracellular space is oxidative. Among those cysteine residues, Cys-592, Cys-603, and Cys-608 are predicted to reside in the extracellular disulfide bond. Namely, the WT ABCG2 is degraded in lysosomes, and the misfolded ABCG2 lacking intramolecular disulfide bond undergoes ubiquitin-mediated protein degradation in proteasomes.

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9 The abbreviations used are: ABC, ATP-binding cassette; CFTR, cystic fibrosis transmembrane conductance regulator; ER, endoplasmic reticulum; FRT, Flp recombination target; PNGase F, peptide N-glycosidase F; TM, transmembrane; WT, wild-type; IP, immunoprecipitation; MW, molecular weight; TM, transmembrane.

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The ABCG2 protein/ABC placenta (BCRP/MXR/ABCP) is reportedly responsible for protecting the body from toxic xenobiotics as well as for rendering cancer cells resistant to anticancer drugs. Furthermore, a potential role of ABCG2 in the regulation of hematopoietic development is suggested (1). In fact, loss of the Abcg2 gene led to a reduction in the number of side population cells in the bone marrow and skeletal muscle (2). Mice lacking Abcg2 displayed a previously unknown type of protoporphyria, where erythrocyte levels of protoporphyrin IX were increased 10-fold (3). We have recently provided direct evidence that human ABCG2 does transport porphyrins across the plasma membrane in an ATP-dependent manner, suggesting a physiological role of ABCG2 in intracellular porphyrin homeostasis (4, 5).

The ABCG2 gene is located on chromosome 4q22, spans over 66 kb, and consists of 16 exons ranging from 60 to 532 bp (6). As compared with the molecular structures of the ABCB1 and ABCC1 transporters, ABCG2 is a so-called “half-transporter” bearing six transmembrane domains and one ATP-binding cassette. Thus, it has been suspected that ABCG2 functions as a homodimer (7–9). We previously demonstrated that human ABCG2 existed in the plasma membrane as a homodimer bound through disulfide-bonded cysteine residues. Treatment with mercaptoethanol reduced the apparent molecular weight of ABCG2 from 140,000 to 70,000 (9). Based on the cDNA sequence, a total of 12 cysteine residues exist in the ABCG2 protein (see Fig. 1). Although the cytosolic space is reductive due to the protection by antioxidants such as glutathione, the extracellular space is oxidative. Among those cysteine residues, Cys-592, Cys-603, and Cys-608 are predicted to reside in the extracellular disulfide bond. Namely, the WT ABCG2 is degraded in lysosomes, and the misfolded ABCG2 lacking intramolecular disulfide bond undergoes ubiquitin-mediated protein degradation in proteasomes.
Cys Role in ABCG2 Protein Stability

A

B

WT C592G C608G

mRNA

Glycosylated
Non-glycosylated
PNGase F treatment

C

MG132 treatment

| Time (h) | WT | C592G | C608G |
|---------|----|-------|-------|
| 0       | 24 | 48    | 0     |
| 0       | 24 | 48    | 0     |
| 0       | 24 | 48    | 0     |

D

Bafirromycin A1 treatment

| Time (h) | WT | C592G | C608G |
|---------|----|-------|-------|
| 0       | 12 | 24    | 0     |
| 0       | 12 | 24    | 0     |
| 0       | 12 | 24    | 0     |

Relative expression level

| Relative expression level |
|---------------------------|
| WT | C592G | C608G |
| 0 h | 24 h | 48 h |
Furthermore, the C592G/C608G variant was mainly localized in intracellular compartments, and its plasma membrane localization was not detectable (10). In this context, it is strongly suggested that the ABCG2 variant lacking the intramolecular disulfide bond is unstable, and its intracellular sorting to the plasma membrane domain is also impaired.

In the present study, we investigated the biochemical importance of the intramolecular disulfide bond formation in the protein stability of ABCG2 by using Flp-In-293 cells in which one single copy of ABCG2 cDNA was incorporated into the designated site of genomic DNA. In this study, we demonstrate that the WT ABCG2 is degraded in lysosomes, whereas misfolded ABCG2 proteins lacking intramolecular disulfide bond undergo ubiquitin-mediated protein degradation in proteasomes. The present study is the first report providing evidence that there are two distinct pathways for protein degradation of ABCG2 WT and mutants lacking the intramolecular disulfide bond.

**EXPERIMENTAL PROCEDURES**

*Cell Culture*—Flp-In-293 cells expressing ABCG2 WT or Cys-to-Gly variants (C592G and C608G) were established and cultured as described previously (10).

*Immunoblot Analysis*—ABCG2-expressing Flp-In-293 cells were placed onto 35-mm dishes and incubated under the above mentioned culture conditions for 24 h. After preincubation, cells were cultured in the presence or absence of compounds at different concentrations for designated incubation periods. After incubation, cells were harvested with their culture medium by centrifugation and washed with ice-cold phosphate-buffered saline. Thereafter, the cells were treated with lysis buffer A containing 50 mM Tris/HCl (pH 7.4), 1% (w/v) Triton X-100, 1 mM dithiothreitol, and protease inhibitor mixture (Complete™, Mini, Nacalai Tesque, Inc., Kyoto Japan). The cell suspension sample was homogenized by passage through a 27-gauge needle and then centrifuged at 800 × g for 10 min at 4 °C.

The resulting supernatant fraction (cell lysate) was subjected to immunoblotting as described previously (10). To enzymatically remove N-linked glycans, when needed, the cell lysate was incubated with PNGase F (New England Biochemicals, Ipswich, MA) at 37 °C for 10 min before the immunoblot analysis.

*Immunoprecipitation*—Following cultivation in the presence or absence of 2 μM MG132 for 24 h, ABCG2-expressing Flp-In-293 cells were harvested by centrifugation and rinsed with ice-cold phosphate-buffered saline. Thereafter, the cells were treated with IP buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% (w/v) Triton X-100, 10 μM MG132, and a protease inhibitor mixture (Complete™, Mini). The samples were homogenized by passage through a 27-gauge needle and then centrifuged at 800 × g for 10 min at 4 °C. Equal amounts of the resulting cell lysate were incubated with preincubated matrix E (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 h at 4 °C with gentle shaking. After centrifugation (17,800 × g, 1 min), the resulting supernatant was used for immunoprecipitation as described below.

The ABCG2 specific monoclonal antibody BXP-21 (Alexis Biochemicals, Lausen, Switzerland) or the anti-ubiquitin monoclonal antibody (Millipore Corp., Billerica, MA) was incubated with preincubated matrix E in Tris-buffered saline with 0.1% (w/v) bovine serum albumin at 4 °C for 1 h with gentle shaking to form the antibody-IP matrix complex. Thereafter, the resulting antibody-IP matrix complex and the preincubated cell lysates incubated were mixed and then further incubated at 4 °C overnight with gentle shaking. Immune complexes thus formed were washed with Tween-Tris-buffered saline, and the subsequently immunoprecipitated proteins were eluted with SDS-PAGE sample buffer containing 2-mercaptoethanol. The resulting elution was subjected to SDS-PAGE and immunoblot analysis.

**RESULTS AND DISCUSSION**

*Protein Expression Levels of ABCG2 WT, C592G, and C608G in Flp-In-293 Cells*—We investigated the role of disulfide bond formation in the protein stability and cellular localization of ABCG2. For this purpose, the Flp-In method was used to integrate cDNA of ABCG2 variants into Flp recombination target-tagged genomic DNA. The expression of endogenous ABCG2 in Flp-In-293 cells was under detection levels in terms of its expression level. Therefore, the Flp-In method was used to integrate cDNA of ABCG2 variants into Flp recombination target-tagged genomic DNA. The expression of endogenous ABCG2 in Flp-In-293 cells was under detection levels in terms of its expression level.
FIGURE 2. MG132 treatment affects expression and function of ABCG2 Cys-to-Gly variants but not WT. A, immunocytochemical staining of Flp-In-293 cells expressing ABCG2 WT, C592G, or C608G. Cells were incubated with or without 2 μM MG132 for 24 h. ABCG2 proteins were immunologically linked with Alexa Fluor 488 (green), and nuclei were stained by propidium iodide (red). Horizontal bars correspond to 20 μm. B, effect of MG132 on ubiquitination of ABCG2 proteins. After incubation with or without 2 μM MG132 for 24 h, cell lysate samples were prepared from Flp-In-293 cells expressing ABCG2 WT, C592G, or C608G. They were then immunoprecipitated with the ABCG2-specific monoclonal antibody (BXP-21) followed by immunoblotting with anti-ubiquitin monoclonal
mRNA and protein, as described previously (10). In the present study, we prepared two ABCG2 variants, C592G and C608G, in which Cys-592 and Cys-608 in the extracellular loop were substituted to Gly, respectively (Fig. 1A). Both fluorescence in situ hybridization mapping and multicolor fluorescence in situ hybridization analysis revealed that ABCG2 cDNA was incorporated into the telomeric region of the short arm on one of chromosomes 12 in Flp-In-293 cells (13). Accordingly, as demonstrated in Fig. 1B, ABCG2 mRNA levels detected by reverse transcription-PCR were almost equal among ABCG2 WT, C592G, and C608G. However, substitution of either Cys-592 or Cys-608 to glycine resulted in a significant decrease in protein levels of ABCG2 in Flp-In-293 cells (Fig. 1B). Both C592G and C608G variants formed homodimers (data not shown; see Fig. 8 in Ref. 10). Immunoblotting without PNGase F treatment revealed two faint bands for each of the C592G and C608G variants, and these bands corresponded to glycosylated (MW = 81,000) and non-glycosylated (MW = 63,000) forms (Fig. 1B). In the case of ABCG2 WT, on the other hand, its glycosylated form (MW = 81,000) appeared to be predominant. After PNGase F treatment, one single band (MW = 63,000) was detected not only for ABCG2 WT but also for the C592G and C608G variants (Fig. 1B). It is suggested that N-linked glycosylation was formed in the C592G and C608G variants as well as the WT in Flp-In-293 cells.

Effect of MG132 or Bafilomycin A1 on the Protein Levels of ABCG2 WT, C592G, and C608G—As shown in Fig. 1C, the protein levels of those ABCG2 variants were remarkably enhanced by the treatment with the proteasome inhibitor MG132. In contrast, the protein level of the ABCG2 WT was not significantly affected by MG132 treatment. The increases in protein levels of the ABCG2 C592G and C608G variants occurred in a time-dependent manner during the MG132 treatment. The treatment with MG132 for 48 h increased the protein level of those variants by 4–5-fold. Importantly, upon the immunoblot analysis with BXP-21 (Fig. 1C, upper panel), aggregated forms (indicated by I) of those variants were detected in the high molecular weight range of over 210,000.

On the other hand, as depicted in Fig. 1D, the protein level of ABCG2 WT increased more than 5-fold when cells were treated with bafilomycin A1, which inhibits lysosomal degradation, whereas the C592G or C608G variants were little affected by the same treatment. No aggregated forms were detected in ABCG2 WT after the 24-h incubation with bafilomycin A1.

Effect of MG132 on the Cellular Localization and Function of ABCG2 WT, C592G, and C608G—It was of great interest to know how the inhibition of proteasomal protein degradation by MG132 affects the cellular localization and functional activity of the ABCG2 C592G and C608G variants. Fig. 2A depicts the immunofluorescence images of Flp-In-293 cells expressing ABCG2 WT, C592G, or C608G that were incubated with or without 2 μM MG132 for 24 h. ABCG2 proteins were probed with the BXP-21 antibody and then labeled with green fluorescence dye (Alexa Fluor 488), whereas DNA in the nuclei was stained with propidium iodide (red fluorescence). Strong green fluorescence was observed at the plasma membrane and intracellular compartments in Flp-In-293 cells expressing ABCG2 WT, which was not affected by MG132 treatment. A remarkable difference was observed after the MG132 treatment, however, in terms of the cellular localization of the C592G or C608G variant proteins. Without MG132 treatment, immunofluorescence of the C592G or C608G variants was extremely weak at the plasma membrane of Flp-In-293 cells (Fig. 2A). Rather, those variant proteins were detected in intracellular compartments, suggesting that the sorting of C592G or C608G variants to the plasma membrane domain was impaired. It is noteworthy, however, that the plasma membrane localizations of the C592G and C608G variants were clearly detected after the MG132 treatment (Fig. 2A).

To investigate the effect of MG132 on the ubiquitinated state of ABCG2 proteins, the Flp-In-293 cells expressing ABCG2 WT, C592G, or C608G were incubated in the presence or absence of 2 μM MG132 for 24 h. As demonstrated in Fig. 2B, significant increases in the ubiquitinated forms of the C592G and C608G variant proteins were detected by immunoblotting with the anti-ubiquitin antibody after immunoprecipitation with the anti-ABC2 antibody BXP-21 (upper panel). In contrast, the ubiquitinated state of the ABCG2 WT protein was little affected by the same MG132 treatment. Similar results were obtained by immunoblotting with BXP-21 after immunoprecipitation with the anti-ubiquitin antibody (lower panel).

As shown Fig. 2C, the MG132 treatment also enhanced cellular resistance to SN-38 in Flp-In-293 cells expressing those variants, whereas little was changed in cells expressing ABCG2 WT even after the MG132 treatment. Based on these results, it is suggested that both C592G and C608G variants that were sorted to the plasma membrane domain after the MG132 treatment were functionally active to extrude SN-38 out of the cells.

Role of Intramolecular Disulfide Bond Formation in Protein Stability—The present study provides evidence that intramolecular disulfide bond formation between Cys-592 and Cys-608 is critically important for stability of the ABCG2 protein. As schematically illustrated in Fig. 2D, this disulfide bond formation is regarded as a key “check point” step that determines the fate of de novo synthesized ABCG2 proteins, namely, either ubiquitination/proteasomal degradation or targeting to the plasma membrane and subsequent lysosomal degradation.

Many proteins of the secretory pathway contain disulfide bonds that are essential for protein folding and function. In the endoplasmic reticulum (ER), rather than relying on small molecule antioxidants such as glutathione (14), it has recently been shown that the disulfide formation is driven by a protein relay
involving endoplasmic reticulum oxidoreductase 1 (Ero1), a novel FAD-dependent enzyme, and protein disulfide isomerase (15, 16). It has not yet been elucidated whether these enzymes are actually involved in the formation of intra- and/or intermolecular disulfide bond in the ABCG2 protein. A significant amount of the ABCG2 protein was detected in intracellular compartments proximal to the nucleus (10). In those compartments, de novo synthesized ABCG2 protein may undergo disulfide bond formation and glycosylation to become the mature protein.

There are two main routes of protein clearance in eukaryotic cells, namely the ubiquitin-proteasome and endosome-lysosome pathways (Fig. 2D). In the present study, we provide evidence that these two distinct pathways play a pivotal role in the protein degradation of ABCG2 WT and mutants lacking the intramolecular disulfide bond. As demonstrated in Fig. 1, C and D, the WT ABCG2 is degraded by the endosome-lysosome pathway, whereas misfolded ABCG2 lacking intramolecular disulfide bond undergoes ubiquitin-mediated protein degradation in proteasomes.

**Protein Quality Control and Protein Degradation Pathways**—The ER is the site where newly synthesized secretory and membrane proteins are folded and assembled under a stringent quality control system, named Check Point in Fig. 2D. Efficient quality control systems have evolved to prevent incompletely folded molecules from moving along the secretory pathway. Accumulation of misfolded proteins in the ER would detrimentally affect cellular functions. Protein folding in the ER is associated with or facilitated by a number of molecular chaperones, including binding protein/GRP78, GRP94, and calreticulin, as well as by folding enzymes such as protein disulfide isomerase and peptidyl-prolyl cis-trans isomerase (17–19). In response to the accumulation of unfolded proteins in the ER, cells activate an intracellular signaling pathway from the ER to the nucleus known as the unfolded protein response, resulting in transcriptional up-regulation of ER-resident proteins as well as ER chaperones (20–23).

Misfolded proteins (e.g. ABCG2 C592G and C608G variants) may be removed from the ER by retrotranslocation to the cytosol and degradation by the ubiquitin-proteasome system (24). This process is known as endoplasmic reticulum-associated degradation. If these misfolded proteins are not degraded in proteasomes (e.g. MG132 inhibition), they are transported in a microtubule-organizing center together with ubiquitin and ER chaperones to form cytoplasmic aggregates called “aggresomes” (25). In fact, ABCG2 appeared to form aggresomes (Fig. 1C, indicated by II) when proteasomal degradation was inhibited by the MG132 treatment in Flp-In-293 cells (Fig. 1C). Aggresome formation was reported for misfolded CFTR proteins as well. Overexpression of CFTR or inhibition of proteasome activity in transfected mammalian cells led to the accumulation of stable, high molecular weight, detergent-insoluble, multiquibrated forms of CFTR (26).

In addition, we anticipate that some part of ubiquitinated ABCG2 C592G and C608G variants can undergo deubiquitination and subsequently be sorted to the plasma membrane domain (Fig. 2D) since these variants were markedly localized at the plasma membrane domain after MG132 treatments (Fig. 2A). The deubiquitination may be catalyzed by ubiquitin-specific processing protease Y (UBPY)/USP8, a deubiquinating enzyme (27, 28). Recently, Mizuno et al. (27) reported that overexpression of UBPY/USP8 reduced the ubiquitination level of epidermal growth factor receptor and delayed its degradation by proteasomes in epidermal growth factor-stimulated cells. Therefore, the fate of ubiquitinated proteins seems to be dynamic, and multiple pathways may exist.

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