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Effect of cysteine mutagenesis on the function and disulfide bond formation of human ABCG2

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Abstract

ABCG2 is a member of the ATP-binding cassette (ABC) transporter superfamily. Its over-expression causes multidrug resistance in cancer chemotherapy. Based on its apparent half size in sequence when compared to other traditional ABC transporters, ABCG2 has been thought to exist and function as a homodimer linked by inter-molecular disulfide bonds. However, recent evidence suggests that ABCG2 may exist as a higher form of oligomers due to non-covalent interactions. In this study, we attempted to create a cystless mutant ABCG2 as a tool for further characterization of this molecule. We found, however, that the cystless mutant ABCG2 is well expressed but not functional. Mapping of the cysteine residues showed that three cysteine residues (C284, C374, and C438) are required concurrently for the function of ABCG2 and potentially for intra-molecular disulfide bond formation. We also found that the cysteine residues (C592, C603, and C608) in the third extracellular loop are involved in forming inter-molecular disulfide bonds and that mutation of these residues does not affect the expression or drug transport activity of human ABCG2. Thus, we conclude that C284, C374, and C438, which may be involved in intra-molecular disulfide bond formation, are concurrently required for ABCG2 function whereas C592, C603, and C608, potentially involved in inter-molecular disulfide bond formation, are not required.

Introduction

Human ABCG2 is a member of the G subfamily of the ATP-binding cassette (ABC) transporter superfamily, which transports a wide variety of substrates (Xu et al., 2007b). Over-expression of ABCG2 has been shown to cause increased drug efflux and resistance in model cancer cell lines (Doyle et al., 1998; Miyake et al., 1999; Allen and Schinkel, 2002). The role of ABCG2 in clinical resistance of human acute myeloid leukemia has also been established (Zhang, 2007). In addition, it has been suggested that ABCG2 plays an important protective role for hematopoietic and cancer stem cells (Zhang, 2007).

Human ABCG2 consists of 655 amino acids (GeneBank: Accession #: Q9UNQ0) which make up one nucleotide binding domain (NBD) at the amino terminal half and one transmembrane domain (TMD) at the carboxyl terminal half of the protein (Fig. 1). Traditional full ABC transporters such as ABCB1 consist of two NBDs and two TMDs. Thus, ABCG2 has been thought to exist and work as a homodimer covalently linked by disulfide bonds (Kage et al., 2002; Litman et al., 2002; Ozvegy et al., 2002). However, it has been found recently that human ABCG2 exists in drug resistant cells primarily as a higher form of oligomer containing 12 subunits with non-covalent interactions (Xu et al., 2004). Electron microscopy examination of

purified human recombinant ABCG2 also revealed that it is a high form of homo-oligomer possibly with 8 identical subunits (McDevitt et al., 2006). It appears that the non-covalent interactions among the subunits in the homo-oligomer are located in the domain including TM5-loop-TM6 (Xu et al., 2007a).

Recently, Henriksen et al. (2005) have demonstrated that one of the cysteine residues (C603) in the third extracellular loop between TM5 and TM6 is responsible for the formation of inter-molecular disulfide bond. Similar observations have been made by Wakabayashi et al. (2006). However, it has also been suggested that inter-molecular disulfide bonds are oxidized during sample preparations (Xu et al., 2004; Bhatia et al., 2005). Furthermore, in the study by Henriksen et al. (2005) it was found that mutation of the cysteine residues (C592 and C608) in the third extracellular loop impaired trafficking and function of ABCG2. Wakabayashi et al. (2007) reported that mutation of these two residues decreased ABCG2 stability. However, Bhatia et al. (2005) observed no effect of similar mutations on the trafficking or function of the mutant form of human ABCG2. Furthermore, Kage et al. (2005) found that mutation of C603 had no effect on ABCG2 function. Thus, it is not yet clear if formation of inter-molecular disulfide bonds is responsible for ABCG2 dimerization and if cysteine residues are important for its membrane targeting and function.

Single nucleotide polymorphisms have also been found to potentially affect human ABCG2 activity. Of 20 known non-synonymous polymorphisms of human ABCG2, none involves a cysteine residue. Interestingly, two non-synonymous mutations, R482G and R482T, resulted in the ability of ABCG2 to transport substrates, such as rhodamine 123, which cannot be transported by the wild type isoform (Han and Zhang, 2004). In both mutation studies by Henriksen et al. (2005) and Bhatia et al. (2005), the mutant human ABCG2^{R482G}, which was found naturally in the drug-selected cancer cells, was used.

Cysless mutant proteins have been powerful tools for studying ABC transporters such as ABCB1 (Loo and Clarke, 1995) and core ABCC1 (Lee and Altenberg, 2003). Because the cysteine residues of human ABCG2 involved in inter-molecular disulfide bond formation may not be essential for ABCG2 function, we tested in this paper the possibility of creating a cysless mutant human ABCG2^{R482G} for use in future studies. We also employed the insect expression system to eliminate the potential problem of folding in mammalian cells for mutant proteins as observed previously for human ABCC7 (Denning et al., 1992). The insect system has been shown to produce high level of active proteins, and suitable for studies such as substrate transport and ATPase activities of wild type and mutant ABC transporters (Bakos et al., 1997; Bakos et al., 2000; Ozvegy et al., 2002). We found that three cysteine residues (C284, C374, and C438) that may be involved in intra-molecular disulfide bond formation are concurrently required for ABCG2 function whereas three cysteine residues (C592, C603, and C608) potentially involved in inter-molecular disulfide bond formation were not required.

Methods

Materials

Insect *Spodoptera frugiperda* SF9 cells, pVL1393 plasmid, and BaculoGold Transfection Kit were purchased from BD Pharmingen. Insect cell culture media and oligonucleotides for site-specific mutagenesis were from Invitrogen. The QuikChange Multisite-Directed Mutagenesis Kit was from Stratagene. Mitoxantrone, rhodamine 123, and Hoechst 33324 were from Sigma. Monoclonal antibody BXP-21 against ABCG2 was from ID Labs. FTC was a gift from Susan Bates (National Cancer Institute). All other chemicals were of molecular biology grade from Sigma or Fisher Scientific

Engineering of ABCG2^{R482G} cDNA in baculovirus vector and multisite-directed mutagenesis

Cysteine wild type full-length human ABCG2^{R482G} cDNA was excised from pcDNA3-ABCG2 (Xu et al., 2004) using Bam HI and Xba I and engineered into pVL1393. To increase expression efficiency, the 5'-UTR of the cDNA was removed by PCR using primers: 5'-CGAGGATCCATGCACCATCACCATCACCATTCTTCCAGTAATGTCGAA-3' (forward) and 5'-GTCTAATCCAGTTGTAGG-3' (reverse). The PCR product lacking the 5'-UTR region was digested with Bam HI and Spe I and used to replace the corresponding regions of ABCG2^{R482G} with the 5'-UTR sequence in pVL1393.

For multisite-directed mutagenesis, full-length ABCG2^{R482G} cDNA was divided into two fragments by Bam HI, Spe I and Xba I, which were then cloned into a pCR-Blunt vector. The resulting constructs, containing the amino- and carboxyl-terminal half-encoding regions, were used as templates to perform site-directed mutagenesis to change cysteines to alanines using the QuikChange Multisite-Directed Mutagenesis Kit according to the manufacturer's instructions. The primers used for specific cysteine to alanine mutations are listed in Table 1. Each fragment containing single or multiple mutations was then used to replace the corresponding wild type sequence in pVL1393. The mutations in the full length ABCG2^{R482G} cDNA were confirmed by sequencing.

Culture of Sf9 cells and infection

Recombinant baculovirus containing cysteine wild type or mutant human ABCG2^{R482G} cDNA were generated with the BaculoGold Transfection Kit according to the manufacturer's instruction. Baculovirus harboring ABCG2 were purified and titrated by plaque assay and end-point dilution. The same MOI were used for all constructs to obtain similar expression levels. Baculovirus containing Xyle was used as a vector control. Uninfected Sf9 cells were cultured in suspension in insect culture medium at 28°C in spinner flasks. Virus-infected Sf9 cells were cultured on 100 mm or 6-well plates.

Western blot and immunofluorescence

Two days after viral infection, the Sf9 cells were harvested, washed once with PBS, and lysed in TNN buffer (50 mM Tris-HCl, pH7.5; 150 mM NaCl; 0.5% Nonidet P-40; 50 mM NaF; 1 mM sodium orthovanadate; 1 mM dithiothreitol; 0.1% SDS and 2 mM phenylmethylsulfonyl fluoride). Next, 20 µg proteins were separated using SDS-PAGE in the absence or presence of 100 mM DTT followed by western blot analysis probed using antibody BXP-21.

Confocal immunofluorescence imaging was conducted as previously described (Yang et al., 2002). Briefly, Sf9 cells were cultured on cover glass in 6-well plates followed by infection with baculovirus containing cysteine wild type or mutant ABCG2^{R482G}. The cells were then fixed with acetone/methanol (1:1) and stained with monoclonal antibody BXP-21 40 hrs after infection. The staining was visualized using FITC-conjugated secondary antibody on a BioRad confocal microscope.

Flow cytometry

Flow cytometry was used to determine the activity of human ABCG2 to transport substrates mitoxantrone, rhodamine 123, or Hoechst 33342 in Sf9 cells as previously described (Ozvegy et al., 2002). Briefly, 40 hrs after infection with ABCG2-containing baculovirus, Sf9 cells were harvested and resuspended in HPMI medium (120 mM NaCl; 5 mM KCl; 400 µM MgCl₂; 40 µM CaCl₂; 10 mM Hepes; 10 mM NaHCO₃; 10 mM glucose and 5 mM Na₂HPO₄). The resuspended cells were preincubated in the presence or absence of 10 µM FTC for 5 min at 37°C followed by a 30-minute incubation at 37°C in the presence of 20 µM mitoxantrone, 1 µM rhodamine 123, or 10 µM Hoechst 33342. The cells were then washed and resuspended in

ice-cold HPMT medium containing 30 $\mu\text{g/ml}$ propidium iodide followed by flow cytometry analysis. Dead cells were excluded based on propidium iodide staining. Relative Activity = $(F_{\text{mutant}} - F_{\text{vector}}) / (F_{\text{wt}} - F_{\text{vector}})$, where F = peak fluorescence intensity of cells due to accumulation of fluorescent drugs.

Results

Characterization of the cysless mutant human ABCG2^{R482G}

It has been shown that mutation of the cysteine residues to alanine in the third extracellular loop (C592, C603, and C608) did not have any effect on ABCG2^{R482G} trafficking and function (Bhatia et al., 2005). This observation forms a basis for testing the possibility of creating a tool, cysless mutant, for studying ABCG2. For this purpose, we mutated all 12 cysteines to alanines (cysless or CL) in ABCG2^{R482G} (Fig. 1). We then expressed the cysless mutant ABCG2^{R482G} along with the cysteine-wild type ABCG2^{R482G} in insect Sf9 cells using the baculovirus expression system. This expression system has previously been used successfully for functional expression of ABCG2 (Ozvegy et al., 2002). As shown in Fig. 2A, both the cysteine-wild type and the cysless mutant ABCG2^{R482G} are equally well expressed and targeted onto plasma membranes in Sf9 cells. However, the cysless ABCG2^{R482G} has little activity in facilitating the efflux of mitoxantrone and rhodamine 123 when compared with the cysteine-wild type ABCG2^{R482G} (Fig. 2B). It appears that the efflux activity observed in Sf9 cells expressing the cysteine-wild type ABCG2^{R482G} is specific to the ectopic human ABCG2^{R482G} because the cells harboring the vector control do not have the efflux activity (Fig. 2C) and the drug efflux activity of the cysteine-wild type ABCG2^{R482G} was sensitive to the ABCG2 inhibitor fumitremorgin C (FTC) (Fig. 2C) as well as GF120918 and novobiocin (data not shown). These observations suggest that mutations of some of the 12 cysteine residues caused the loss of ABCG2^{R482G} function.

Mapping cysteine residues that are functionally important

To determine which of the 12 cysteine mutations is likely responsible for the loss of function in the cysless mutant ABCG2^{R482G}, we used three constructs with mutations of cysteine residues in various domains of ABCG2^{R482G}. These constructs were named C9-CL, I5-CL, and C4-CL as shown in Fig. 3A. Fig. 3B shows that all these three cysteine-mutant ABCG2's could be successfully expressed and targeted onto plasma membranes. This observation is expected since the CL mutant without any cysteine residues could be successfully expressed in Sf9 cells (Fig. 2). Interestingly, only the C4-CL mutant retained the majority of its mitoxantrone efflux activity while both the C9-CL and I5-CL mutants lost most of these activities. These observations suggest that the functionally important cysteine residue(s) may include all or partially the internal five cysteine residues (C284, C374, C438, C491, and C544), which are commonly mutated in both the non-functional C9-CL and I5-CL, but are intact in the functional C4-CL.

To verify if C284, C374, C438, C491, and C544 residues are important for ABCG2^{R482G} function, we engineered another construct (N3C4-CL, Fig. 4A) which maintains all of these five cysteine residues, but has all the remaining cysteines mutated to alanine. This mutant is well expressed (Fig. 4B) and retains ~80% of the mitoxantrone efflux activity of the cysteine-wild type ABCG2^{R482G} (Fig. 4C). Further mutation of N3C4-CL by changing C284, C374, and C438 to alanines (construct N6C4-CL, Fig. 4A) completely eliminated the mitoxantrone efflux activity of human ABCG2^{R482G} (Fig. 4C) although they are also well expressed. Thus, it is possible that the functionally important cysteine residues are C284, C374, and C438.

To map which of the three cysteine residues are functionally important, we engineered four more constructs by mutating all or partial of the three cysteines C284, C374, and C438 to

alanines and generated constructs I3-CL, I2-CL, C284A, and C374A (see Fig. 5A). All these cysteine-mutant ABCG2^{R482G} constructs could be successfully expressed (Fig. 5B). However, only I3-CL with all three cysteines mutated to alanine lost the majority of its mitoxantrone efflux activity (Fig. 5C). Mutation of one or two of these residues (I2-CL, C284A, and C374A) did not significantly affect the ABCG2^{R482G} activity to transport mitoxantrone. I3-CL with C284A, C374A, and C438A mutations also lost most of its ability to transport another substrate, Hoechst 33342 (Fig. 5D). These observations suggest that C284, C374, and C438 need to work together for the function of ABCG2^{R482G}. In addition, these three cysteines do not appear to affect the substrate specificity.

Cysteine residues that are involved in the formation of intra- and inter-molecular disulfide bond

It has been thought previously that the formation of inter-molecular disulfide bonds is important for ABCG2 homodimerization (Kage et al., 2002; Litman et al., 2002; Ozvegy et al., 2002). Because we have several ABCG2^{R482G} constructs with mutations of various cysteine residues, it would be interesting to determine if any of these mutations, especially the ones that eliminated ABCG2^{R482G} activity, affect disulfide bond formation as observed on non-reducing SDS-PAGE. As shown in Fig. 6B, the cysteine-wild type ABCG2^{R482G} did show some dimeric molecules under the non-reducing condition (without DTT). However, the dimeric ABCG2^{R482G} represents only a small fraction of total ABCG2^{R482G} detected. We also observed that the dimeric ABCG2^{R482G} band is broad (probably with 3 different populations), likely due to the existence of different intra-molecular disulfide bonds, which potentially cause generation of different shapes of molecules with different mobility on the non-reducing SDS-PAGE (Urbatsch et al., 2001) (see also below).

The disulfide-bond-linked dimeric ABCG2^{R482G} on the non-reducing SDS-PAGE is completely eliminated with mutations in the CL and C9-CL (Fig. 6B, lanes 2 and 3). The dimeric ABCG2^{R482G} on non-reducing SDS-PAGE was observed with the I5-CL mutant (Fig. 6B, lane 4). Because (a) C9-CL has only three cysteines at the amino terminus (C43, C55, C119) and does not form any dimers and (b) I5-CL has seven cysteines with three at the amino terminus, same as C9-CL (C43, C55, C119), four at the carboxyl terminus (C592, C603, C608, and C635) and could form dimers of fast mobility, we conclude that the formation of the inter-molecular disulfide bond likely requires the last four cysteine residues (see Fig. 3A). In light of previous findings (Bhatia et al., 2005; Henriksen et al., 2005), we propose that the formation of the inter-molecular disulfide bond is likely to require the cysteine residues located in the third extracellular loop linking TM5 and TM6. To test this hypothesis, we engineered another construct which has all three cysteine residues in the third extracellular loop mutated to alanine (C592A, C603A, and C608A) in order to determine if dimers linked by inter-molecular disulfide bonds exist with this mutant. As shown in Fig. 6C (lane 3), this mutation (construct L3-CL, see Fig. 6A) essentially eliminated dimers on the non-reducing SDS-PAGE. Thus, it is likely that the cysteines in the third extracellular loop are responsible for the formation of inter-molecular disulfide bonds.

It is interesting to note, however, that the non-reducing SDS-PAGE profile of the dimeric I5-CL mutant is different from the cysteine-wild type ABCG2^{R482G} (compare lanes 1 and 4, Fig. 6B). While the dimeric cysteine-wild type ABCG2^{R482G} band is broad possibly with 3 different populations, the I5-CL appears to have only a distinct one dimer band of fast mobility on the non-reducing SDS-PAGE (lane 4, Fig. 6B). This difference may be due to the mutation of other cysteine residues in the I5-CL which could potentially affect the formation of intra-molecular disulfide bonds and, thus, the shape of the molecule and mobility on the non-reducing SDS-PAGE. To test this possibility, we studied other mutant I2-CL, C284A, and I3-CL using the non-reducing SDS-PAGE. As shown in Fig. 6C, the mutant I3-CL had a single dimeric

ABCG2^{R482G} band of fast mobility on the non-reducing SDS-PAGE (lane 8), similar to the I5-CL mutant (see Fig. 6A). However, the mutant C284A had an additional dimeric band of medium mobility whereas the I2-CL has an additional dimeric band of slow mobility (Fig. 6C, lanes 6 and 7). The dimeric bands with slow and medium mobility may represent dimeric ABCG2^{R482G} containing both inter- and intra-molecular disulfide bonds. Because the mutant construct C284A has the wild type C374 and C438 residues and both mutants I2-CL and I3-CL have these two cysteine residues mutated, it is possible that these two cysteines are involved in the formation of intra-molecular disulfide bonds which result in the dimeric protein of medium mobility on non-reducing SDS-PAGE. Because the difference between I2-CL and I3-CL is that C284 is mutated in the later, but not the former, it is possible that the dimeric band of slow mobility observed with I2-CL may be due to the existence of C284. It is also noteworthy that this dimeric band of slow mobility was also observed, albeit at low level, with the mutants C4-CL (Fig. 6B, lane 5) and L3-CL (Fig. 6C, lane 3) which contain wild type C284, but not with C9-CL and I5-CL which have a mutated C284, consistent with the conclusion that C284 may be responsible for the production of the dimeric protein of slow mobility on the non-reducing SDS-PAGE.

Discussion

In this study, we have created a cysless mutant of human ABCG2^{R482G} and shown that mutating all 12 cysteine residues effectively eliminated ABCG2^{R482G} activity. Further mapping studies showed that three cysteine residues (C284, C374, and C438) are essential for human ABCG2^{R482G} function. However, mutating these residues, unless concurrently, did not affect ABCG2^{R482G} function. Previously, Kage et al. (2005) also found that single mutation of any of these cysteine residues did not affect ABCG2 transport activity. However, Kage et al. (2005) did not perform a study using mutations in combination. Based on these findings, we conclude that three cysteine residues (C284, C374, and C438) are required concurrently for ABCG2^{R482G} activity.

Human ABCG2 has 12 cysteine residues with five in the amino terminal NBD and seven in the carboxyl terminal TMD. The three functionally important cysteine residues (C284, C374, and C438) are located in the center of the molecule with C284 and C374 in the NBD and C438 in the TMD. Both C284 and C374 are not directly located in the Walker A (amino acid residues 80–88) or B (residues 206 to 211) motifs and, thus, may not be directly involved in affecting nucleotide binding. The observation that the single mutation of these two cysteine residues (C284A and C374A) did not substantially affect ABCG2^{R482G} activity, confirms that their mutations did not severely influence the nucleotide-binding activity. The third residue C438 is located in TM2. Mutation of C438 to alanine along with C374 (construct I2-CL) did not affect ABCG2^{R482G} activity. C438A mutation alone also did not affect ABCG2^{R482G} function (Kage et al., 2005). Hence, these three cysteine residues need to work together to provide the protein with functionality.

The results shown in Fig. 6 also suggest that the C284, C374 and C438 residues may be involved in formation of intra-molecular disulfide bonds. Mutating C374 and C438 (construct I2-CL) effectively eliminated formation of the dimeric protein of medium mobility on non-reducing SDS-PAGE whereas mutating C284 eliminated the dimeric protein of slow mobility. It is, however, not yet known if the intra-molecular disulfide bond is important for ABCG2^{R482G} function. Considering the fact that all these three cysteine residues are functionally important, the formation of the potential intra-molecular disulfide bonds by these residues may be important for the ABCG2^{R482G} function. Clearly, further studies are needed to determine if the disulfide bonds formed by these three residues play any role in ABCG2 function.

We also found that three cysteine residues in the third extracellular loop (C592, C603, and C608) are likely involved in the formation of inter-molecular disulfide bonds, consistent with previous findings (Bhatia et al., 2005; Henriksen et al., 2005; Kage et al., 2005). However, we found no evidence that these three cysteine residues are required for the expression and drug efflux activity of human ABCG2^{R482G}, in contrast to observations by Henriksen et al. (2005), but consistent with that by Bhatia et al. (2005) and Kage et al. (2005). The reason for the discrepancy among these studies with respect to the effect of mutations on ABCG2^{R482G} function is not yet known. The only difference between these studies is the expression system used, which may or may not be the cause of the difference in function. While HEK293 cells were used by Henriksen et al. (2005), HeLa, PA317, and Sf9 cells were used by Bhatia et al. (2005), Kage et al. (2005), and in our study, respectively.

It is noteworthy, however, that the dimers linked by inter-molecular disulfide bonds observed on the non-reducing SDS-PAGE represent only a very small fraction of ABCG2^{R482G} expressed in this study. The vast majority of ABCG2^{R482G} does not have inter-molecular disulfide bonds. It also appears that the cysteine wild-type ABCG2^{R482G} has several species of dimers on the non-reducing SDS-PAGE, suggesting the existence of several molecules with different intra-molecular disulfides (see discussion above). Previously, it has been observed that the formation of inter-molecular disulfide bonds may be due to oxidation during sample preparation (Xu et al., 2004). Based on these observations and the finding that elimination of inter-molecular disulfide bonds by mutations does not affect ABCG2^{R482G} activity, we conclude that these inter-molecular disulfide bonds of ABCG2^{R482G} may not exist *in vivo* and may not be necessary for the transport function of ABCG2^{R482G}.

In summary, we have shown in this study that, unlike ABCB1 (Loo and Clarke, 1995) and core ABCC1 (Lee and Altenberg, 2003), a cystless mutant of human ABCG2 is not functional and, thus, cannot be used as a tool for future studies. Through this study, we have identified three functionally important cysteine residues (C284, C374, and C438) which may need to work together to sustain the functionality of human ABCG2^{R482G}. Furthermore, the cysteine residues in the third extracellular loop are responsible for the minor population of dimeric ABCG2^{R482G} molecules observed on non-reducing SDS-PAGE and their mutations do not affect ABCG2 function.

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Abbreviations

ABC, ATP-binding cassette; TMD, transmembrane domain; NBD, nucleotide-binding domain; CL, cystless.

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Figure 1.

Schematic topological and linear structure of ABCG2 with cysteine residues indicated. ABCG2 consists of 6 transmembrane segments (boxes) and 12 cysteine residues (solid balls) with both its amino and carboxyl termini located in cytoplasm. The cysteine residues indicated with asterisks are functionally important while the underlined ones are important for putative intermolecular disulfide bond formation.

Figure 2.

Expression and function of wild type and cysless mutant ABCG2^{R482G} in insect Sf9 cells. A, western blot and confocal immunofluorescence analyses of Sf9 cells expressing Xyle (Vec control), wild type (WT) or cysless (CL) mutant ABCG2^{R482G}. B, accumulation of mitoxantrone and rhodamine 123 in Sf9 cells expressing Xyle, wild type or cysless mutant ABCG2^{R482G}. C, effect of ABCG2 inhibitor FTC on mitoxantrone accumulation in Sf9 cells expressing Xyle, wild type or cysless mutant ABCG2^{R482G}. The baseline was the auto fluorescence of cells in the absence of drug substrates.

Figure 3.

Expression and function of ABCG2^{R482G} with cysteine mutations in three different domains. A, schematic linear structure of wild type and mutant ABCG2^{R482G} with mutated cysteines in three different domains. B, western blot and confocal immunofluorescence analyses of Sf9 cells expressing the three mutant ABCG2^{R482G}, C9-CL, I5-CL, and C4-CL. C, relative activity of mutant ABCG2^{R482G}, CL, C9-CL, I5-CL, and C4-CL compared with wild type ABCG2^{R482G} as determined using flow cytometry for mitoxantrone accumulation in Sf9 cells. Data shown are from 4 independent experiments with standard deviation.

Figure 4.

Mapping the functionally important cysteine residues. A, schematic linear structure of wild type and mutant ABCG2^{R482G}. B, western blot analyses of Sf9 cells expressing XylE, wild type and mutant ABCG2^{R482G}. C, relative activity of mutant ABCG2^{R482G}, N3C4-CL and N6C4-CL, compared with wild type and cystless (CL) ABCG2^{R482G} as determined using flow cytometry for mitoxantrone accumulation in Sf9 cells expressing these ABCG2's. Data shown are from 3 independent experiments with standard deviation.

Figure 5.

Fine tuning the functionally important cysteine residues and substrates selectivity. A, schematic linear structure of wild type and mutant ABCG2^{R482G}. B, western blot analyses of Sf9 cells expressing Xyle, wild type and mutant ABCG2^{R482G}. C, relative activity of mutant ABCG2^{R482G} I2-CL, C284A, I3-CL, and C374A compared with wild type and cystless (CL) ABCG2^{R482G} as determined using flow cytometry for mitoxantrone (MX) accumulation in Sf9 cells. Data shown are from 3 independent experiments with standard deviation. D, effect of C284A, C374A, and C438A mutations (construct I3-CL) on efflux of Hoechst 33342 (Hoechst) as determined using flow cytometry. Data shown are from 4 independent experiments with standard deviation.

Figure 6.

Reducing and non-reducing SDS-PAGE analyses of wild type and cysteine mutant ABCG2^{R482G}. A, schematic linear structure of wild type and mutant ABCG2^{R482G} with cysteine mutations in three different domains. B, reducing and non-reducing SDS-PAGE of wild type and cysteine mutant ABCG2^{R482G} CL, C9-CL, I5-CL, and C4-CL. C, non-reducing SDS-PAGE of cysteine mutant ABCG2^{R482G} constructs L3-CL, I2-CL, C284A, and I3-CL compared with wild type and cysless mutant, CL.

Table 1

Primers used for construction of cysless mutants

Mutations	Primer sequence	RES ^a
C43A	TTTCATAACATTGCCTATCGAGTAAACTGAAG	BsrD I
C55A	GCTTTCTACCTGCACGAAAACAGTTGAG	Bsg I
C119A	GCCAATTTCAAAGCGAATTCAGGTTACGTGG	EcoR I
C284A	GAATCAGCTGGATATCACGCTGAGGCCTATAATAAC	EcoR V
C374A	ACACCACCTCCTTCGCTCATCAACTCAGATG	none
C438A	CTGACGACCAACCAAGCTTTCAGCAGTGTTTC	Hind III
C491A	TATATTTACCGCTATAGTATACTTCATGTTAGG	Acc I
C544A	CTTCTCATGACGATCGCTTTTGTGTTTATGATG	Pvu I
C592A	GGACAAAACCTCGCCCCGGGACTCAATGCAA	Sma I
C603A/C608A	AGGAAACAATCCTGCTAACTATGCAACAGCTACTGGCGAAGAATATTT	-Nsp I
C635A	CACGTGGCCTTGGCTGCAATGATTGTTATTTTC	BsrD I

^aRestriction (RES) enzyme digestion sites engineered in the primer for the convenience of detection. The primer sequence for C603A/C608A mutant does not contain the Nsp I site present in the wild type sequence.