Mutational Analysis of ABCG2: Role of the GXXXG Motif

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Received January 28, 2004; Revised Manuscript Received May 14, 2004

ABSTRACT: ABCG2 (BCRP/MXR/ABCP) is a half-transporter associated with multidrug resistance that presumably homodimerizes for function. It has a conserved GXXXG motif in its first transmembrane segment, a motif that has been linked with dimerization in other proteins, e.g., glycophorin A. We substituted either or both glycines of this GXXXG motif with leucines to evaluate the impact on drug transport, ATP hydrolysis, cross-linking, and susceptibility to degradation. All mutants also carried the R482G gain-of-function mutation, and all migrated to the cell surface. The mutations resulted in lost transport for rhodamine 123 and impaired mitoxantrone, pheophorbide *a*, and BODIPY-prazosin transport, particularly in the double leucine mutant (G406L/G410L). Basal ATPase activity of the G406L/G410L mutant was comparable to the empty vector transfected cells with no substrate induction. Despite impaired function, the mutants retained susceptibility to cross-linking using either disuccinimidyl suberate (DSS) or the reducible dithiobis-(succinimidyl propionate) (DSP) and demonstrated a high molecular weight complex under nonreducing conditions. Mutations to alanine at the same positions yielded fully functional transporters. Finally, we exposed cells to mitoxantrone to promote folding and processing of the mutant proteins, which in the leucine mutants resulted in increased amounts detected on immunoblot and by immunofluorescence. These studies support a hypothesis that the GXXXG motif promotes proper packing of the transmembrane segments in the functional ABCG2 homodimer, although it does not solely arbitrate dimerization.

The role of ATP-binding cassette (ABC)[1] transporters in mediating multidrug resistance in cancer has been studied for over 20 years (*1*). With 48 known members classified in seven subgroups (A through G), ABC transporters constitute the largest family of human cellular transporters (*2*). ABCG2, also called MXR/BCRP/ABCP (*3*−*5*), is a 655 amino acid membrane protein that confers resistance to mitoxantrone (*3*, *6*−*8*), flavopiridol (*8*), methotrexate (*9*), and the camptothecin derivatives SN-38 (*8*, *10*) and topotecan (*8*, *11*). Comprised of one transmembrane domain with six predicted transmembrane segments (TMs) and a single ATP-binding domain, ABCG2 is considered a "half-transporter", like the other members of the G subfamily of human ABC transporters. Early studies with bacterial ABC transporters demonstrated that a functional transporter requires two ATP-binding domains coupled to two transmembrane domains, each typically containing six membrane-spanning segments (*12*). The entire transporter may be comprised in a single polypeptide chain, such as the human P-glycoprotein (*13*), may be pieced together from as many as four molecules, as in the bacterial transporter Drr (two DrrA and two DrrB subunits) (*14*), or may also be formed as a heterodimer of two half-transporters, as in the case of ABCG5 and ABCG8 (*15*).

A 72 kDa protein on SDS−PAGE, it is widely accepted that ABCG2 homodimerizes to generate a functional transporter. This assessment is based on transfection studies with the single subunit that creates, in contrast to ABCG5 and ABCG8, a functional transporter expressed on the cell surface. This has been demonstrated in mouse fibroblasts (*16*), HeLa cells (*17*), MCF-7 human breast cancer cells (*4*), and Sf9 insect cells (*18*). The molecular interactions and amino acid residues that control this dimerization process are largely unknown. Kage et al. reported that disulfide bonds were candidates for mediating ABCG2 homodimerization, since electrophoresis under nonreducing conditions resulted in high molecular weight bands, consistent with dimers or multimers (*19*). However, work in several model systems has suggested that cysteines do not play a critical role in folding or in dimerization of ABC transporters. For example, the bacterial half-transporter LmrA has no cysteines at all (*20*), yet its substrate transport was confirmed (*21*).

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[1] Abbreviations: BCRP, breast cancer resistance protein; MXR, mitoxantrone resistance protein; ABCP, ABC transporter expressed in placenta; ABC, ATP-binding cassette; TM, transmembrane segment; DTT, dithiothreitol; DSS, disuccinimidyl suberate; DSP, dithiobis-(succinimidyl propionate); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; HEK, human embryonic kidney; FTC, fumitremorgin C; MX, mitoxantrone.

10.1021/bi0497953
Published on Web 07/02/2004

In case of the ABCG5 and ABCG8 heterodimer, both half-transporters must be present in order to express the protein on the cell surface (*15*). Mutations have been identified in several half-transporters that render the protein unable to dimerize. Mutations in TMs of the *Drosophila* white family (an orthologue of the human ABCG subfamily) have been postulated to interfere with dimerization (*22*).

ABC transporters have a high degree of identity in their nucleotide binding domains but show relatively little homology in the transmembrane domains (*2*). Consequently, it is difficult to identify essential structural elements of TMs based on primary structure conservation. Recently obtained crystal structures have identified putative dimer interfaces in bacterial ABC transporters, e.g., the lipid A-flippase MsbA (*23*, *24*). However, the relative positioning of the TM domains in the primary structure of the ABCG subfamily is different from that of MsbA or that of P-glycoprotein. Transmembrane segments in these latter transporters are positioned near the N-terminus of homologous halves, while they occupy a C-terminal position in the ABCG half-transporters. Therefore, it is possible that members of the ABCG subfamily of transporters have significantly different packing of TMs compared to MsbA or P-glycoprotein. Thus, it is notable that the G/A/SXXXG motif is surprisingly conserved in TM1 of members of the ABCG subfamily.

The GXXXG motif has been identified as one of the most frequently occurring transmembrane sequence motifs and as a potential site for tight interaction between TM α-helices (*25*, *26*). The four-residue separation aligns the glycines of the GXXXG motif on one face of the helix, providing a flat platform for close binding to the other helix. GXXXG has been linked with dimerization in proteins such as glycophorin A (*27*), human carbonic anhydrase (*28*), Ff bacteriophage major coat protein (*29*), yeast ATP synthase (*30*), *Helicobacter pylori* vacuolating toxin (*31*), yeast α-factor receptor (*32*), and members of the epidermal growth factor receptor (ErbB) family (*33*). In ABCG2, TM1 is the only transmembrane segment containing a GXXXG motif. We show that substitution of the two glycines in the GXXXG motif with leucine residues results in a protein that retains its expression on the cell surface, retains sensitivity to cross-linking, but has impaired ATP hydrolysis and substrate transport. Further, results are presented that suggest improper folding and increased degradation of these mutants.

EXPERIMENTAL PROCEDURES

Cell Culture. Human embryonic kidney (HEK) 293 cells (ATCC, Manassas, VA) were maintained in Eagle's minimal essential medium (ATCC) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 2 mM glutamine (BioFluids, Rockville, MD), and 100 units/L penicillin/streptomycin (BioFluids) at 37 °C in 5% CO_2. Transfected cell lines were grown in 2 mg/mL G418 (Invitrogen).

ABCG2 transfected HEK293 cells (at 80−90% confluence) were treated overnight with 2−10 μM mitoxantrone (Sigma, St. Louis, MO) followed by microsomal membrane preparation as described below.

Generation of Mutants. The GXXXG mutants were generated by site-directed mutagenesis in a pcDNA3.1 vector (Invitrogen) carrying a mutation at amino acid 482 in ABCG2. This vector was derived from S1-M1-80 cells, a mitoxantrone-resistant human colon carcinoma cell line, which encodes a glycine at amino acid 482 instead of the wild-type arginine (*17*). This vector was selected for ease of study with rhodamine 123, a substrate for ABCG2 only when the R482G mutation is present.

The following ABCG2 mutants were generated: G406L, G410L, G406L/G410L, G406A, G410A, and G406A/G410A. For construction of the pcDNA3.1/Myc-HisA(−)-MXRa mutant vectors we have designed four primers (MXRa 5′, MXRa 3′, MXR406/410 3′, and MXR406/410 3′) for each mutant. MXRa 5′ contains a *Not*I site, Kozak sequence, and ATG start codon of the MXR gene, while MXRa 3′ contains a stop codon and *Bam*HI site. MXR406/410 5′ and MXR406/410 3′ contain the mutation sites of amino acids 406 and 410 (glycine to leucine or alanine at either or both sites). For each mutant we have performed two primary PCRs using pcDNA3.1/Myc-HisA(−)-MXRa as the DNA template with the appropriate MXRa 5′/MXR406/410 3′ and the MXRa 3′/ MXR406/410 5′ primer sets. PCR was performed by the Stratagene protocol. The 1250 and 770 bp fragments generated by the primary PCR were annealed and used as the template for the secondary PCR with the MXRa 5′/MXRa 3′ primer set. The 2 kb fragment from the secondary PCR and pcDNA3.1/Myc-HisA(−)-MXRa was digested with *Not*I and *Bam*HI sequentially. The mutations were confirmed by DNA sequencing. The 2 kb insert and 5.5 kb vector were then gel isolated and ligated together.

Transfection. Stable transfectants were generated in human embryonic kidney (HEK) 293 cells. Cells were plated in 100 mm cell culture dishes 24 h before transfection (750000 cells/dish). Transfections were performed by incubating the cells at 37 °C in 5% CO_2 for 1 h in 6 mL of medium containing a 1:2 ratio of plasmid DNA and TransFast transfection reagent (Promega, Madison, WI). Cells were then incubated for an additional 48 h in medium containing 10% fetal bovine serum before the addition of 2 mg/mL G418. Cultures were carried out in 2 mg/mL G418 with frequent removal of dead cells until resistant colonies appeared. Colonies were then isolated using trypsinized sterile cloning discs (PGC Scientific, Frederick, MD), and those positive for ABCG2 with the surface antibody 5D3 (eBioscience, San Diego, CA) were expanded prior to study.

Microsomal Membrane Preparation. After being washed twice in ice-cold DPBS, cells were scraped into hypotonic lysis buffer [5 mM EGTA, 5 mM EDTA, 10 mM Tris-HCl, pH 7.4, 10 mM HEPES, 5 μg/mL aprotinin, 5 μg/mL leupeptin, and 2 mM PMSF, plus or minus 2 mM dithiothreitol (DTT)] and disrupted by nitrogen cavitation (Parr Instrument, Moline, IL) for 20 min. Nuclei were sedimented by centrifugation at 1200 rpm for 10 min, mitochondria were removed by centrifugation at 7000 rpm for an additional 10 min, and finally the microsomal fraction was sedimented in a centrifugation at 40000 rpm for 1 h. Samples were kept at 4 °C for the duration of the entire procedure. The pellets were resuspended in lysis buffer and stored at −20 °C.

Immunoblot Analysis. Protein concentrations were measured by the Bradford method with Bio-Rad's protein assay reagent (Bio-Rad, Hercules, CA) using BSA standards (Pierce, Rockford, IL). Membranes (15−25 μg) were loaded onto a 7.5% Tris-HCl gel (Bio-Rad) in 2% SDS loading buffer and subjected to electrophoresis. For nonreducing conditions β-mercaptoethanol was omitted from the loading

buffer. Proteins were then transferred to Immobilon membrane (Millipore, Bedford, MA) and after being blocked in TBS with 5% milk membranes were incubated overnight at 4 °C with a 1:250 dilution of the BXP-21 monoclonal antibody (Kamiya Biomedical, Seattle, WA). Membranes were washed four times in TBS/milk containing 0.05% Tween; then a 1:2000 dilution of anti-mouse secondary horseradish peroxidase-conjugated antibody (Amersham, Piscataway, NJ) was applied for 1 h at room temperature, followed by enhanced chemiluminescence detection (ECL Western blotting detection reagent; Amersham). Prior to incubation with the primary antibody membranes were stained in 5% acetic acid containing 0.1% Ponceau S (Sigma) for 10 min and checked for comparable loading.

Northern Blot Analysis. RNA was extracted from cells using RNA STAT-60 (Tel-Test Inc., Friendswood, TX) according to the manufacturer's instructions. Northern blot analysis was performed by standard methods. Labeling of cDNAs was accomplished using Riboprobe in vitro transcription systems (Promega). To compare the quality and quantities of RNA, 5 μg of total RNA was electrophoretically separated in a 1% agarose/6% formaldehyde gel. Gels were stained with ethidium bromide and checked for comparable loading. Northern blot labeling was performed using a riboprobe generated from the first 662 bp of ABCG2 subcloned in a pCRII-TOPO vector (Invitrogen).

Flow Cytometry. For studies with the anti-ABCG2 antibody 5D3, cells were trypsinized and resuspended in DPBS with 2% BSA to which phycoerythrin-conjugated 5D3 or phycoerythrin-conjugated mouse IgG was added. The cells were incubated with antibody for 30 min at room temperature, washed twice with DPBS, and kept in the dark until analyzed. For the transport studies, cells were trypsinized, resuspended in complete media [phenol red-free IMEM with 10% fetal calf serum] containing 20 μM mitoxantrone (Sigma), 0.5 μg/mL rhodamine 123 (Sigma), 10 μM pheophorbide *a* (Frontier Scientific, Logan, UT), or 250 nM BODIPY-prazosin (Molecular Probes, Eugene, OR) with or without 10 μM of the ABCG2 blocker fumitremorgin C (FTC), and incubated for 30 min at 37 °C in 5% CO_2. (FTC was synthesized by Thomas McCloud, Developmental Therapeutics Program, Natural Products Extraction Laboratory, National Institutes of Health, Bethesda, MD.) Cells were then washed once in cold complete medium and then incubated for 1 h at 37 °C in substrate-free media, continuing with or without 10 μM FTC. Subsequently, cells were washed twice with cold DPBS and placed on ice in the dark until analyzed. Cells were analyzed on a FACSort flow cytometer, equipped with both a 488 nm argon laser and a 635 nm red diode laser. For all samples, at least 10000 events were collected. Debris was eliminated by gating on forward versus side scatter, and dead cells were excluded on the basis of propidium iodide staining.

Cross-Linking. Chemical cross-linking was performed in vivo on intact cells (*34*). After incubation at room temperature for 30 min with the cross-linking agents disuccinimidyl suberate (DSS) (Pierce) or dithiobis(succinimidyl propionate) (DSP) (Pierce) at 2 mM final concentration, the reaction was terminated by addition of Tris-HCl (pH 8) to 20 mM, and cells were immediately harvested as described above.

ATP Hydrolysis Assay. ATPase activity of ABCG2 in crude membranes was measured by the end point, P_i assay as previously described (*35*). Crude membranes were prepared as described previously and stored at −70 °C in TSNa buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 250 mM sucrose, 1 mM AEBSF, and 1% aprotinin) (*36*). ABCG2-specific activity was recorded as the vanadate-sensitive ATPase activity. The assay measured the amount of inorganic phosphate released over 20 min in the ATPase assay buffer (10 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM dithiothreitol, 2 mM EGTA, 5 mM sodium azide, and 1.5 mg/mL ouabain) at 37 °C. The assay was carried out under basal conditions or in the presence of increasing concentrations of prazosin (0, 1, 10, and 100 μM). The reaction was initiated with 5 mM ATP and quenched with SDS (2.5% final concentration); the amount of P_i released was quantitated using a colorimetric method.

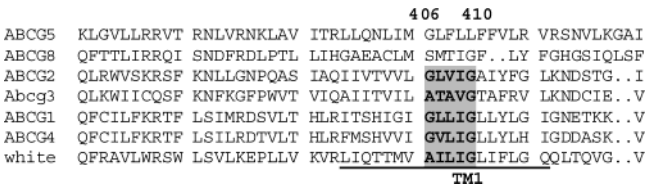

FIGURE 1: Sequence alignment for members of the ABCG subfamily. Note the conserved GXXXG or AXXXG sequence motifs in TM1 of members of the human G subfamily, the murine Abcg3, and the *Drosophila* white protein.

Immunofluorescence. For confocal microscopy cells were plated onto 12 mm cover glasses at a density of 1.5×10^5 cells/cover glass and cultured for 2 days. After one brief wash with Dulbecco's modified PBS, cells were fixed for 5 min at room temperature with 4% paraformaldehyde in DPBS. After five washes with DPBS, samples were further fixed and permeabilized with prechilled (−20 °C) methanol for 5 min at room temperature. After five additional washes with DPBS, cells were blocked for 1 h at room temperature in DPBS-based blocking buffer containing 2 mg/mL bovine serum albumin (ICN, Aurora, OH), 1% fish gelatin (Goldmark Biologicals, Phillipsburg, NJ), 0.1% Triton X-100 (Sigma), and 5% goat serum (Vector Laboratories, Burlingame, CA). Samples were then incubated with a 1:100 dilution of the mouse monoclonal anti-ABCG2 antibody BXP-21 (Kamiya) for 1 h at room temperature. After subsequent washes, cells were incubated for 1 h at room temperature with a 1:250 dilution of fluorescein horse anti-mouse IgG (Vector Laboratories).

RESULTS

GXXXG Motif Mutants. ABCG2 is a half-transporter that is thought to homodimerize for function; a potential site for such interaction in membrane proteins is the GXXXG dimerization motif. Sequence alignment of the G subfamily of human ABC transporters (Figure 1) illustrates a GXXXG motif in the first TM segment of ABCG1, ABCG2, and ABCG4. In addition, the *Drosophila* white gene and the murine Abcg3 contain an AXXXG motif at this position, a motif that has also been reported to be among the most prevalent sequence motifs in TM α-helices (*37*). Alanine, only one methyl group larger than glycine, is also found in the AXXXA motif, which, just like GXXXG, occurs at a significantly higher frequency above the expected in α-helices and is implicated in thermostability of protein structures (*38*).

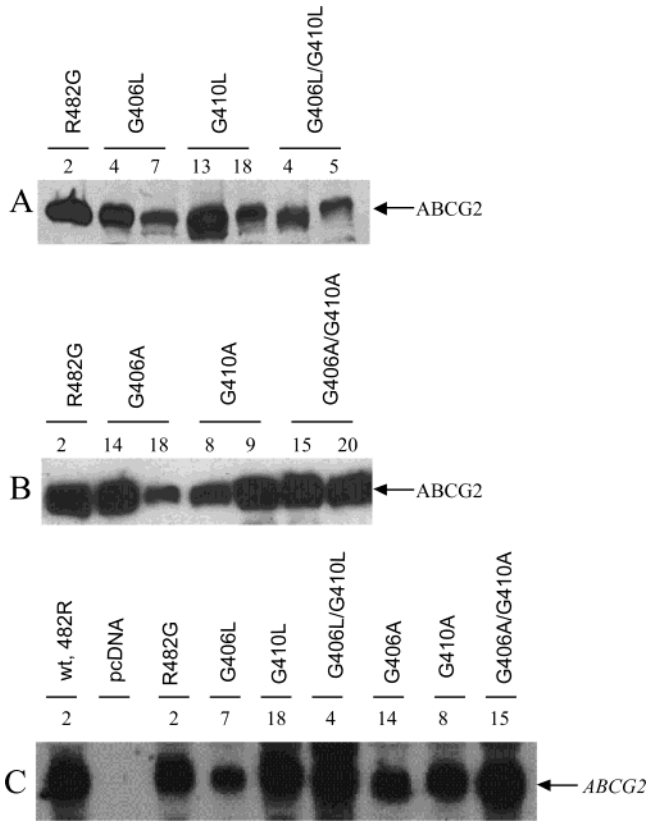

FIGURE 2: Protein and RNA levels of the GXXXG mutants transfected into HEK293 cells. (A) Protein expression levels on immunoblot with the BXP21 monoclonal anti-ABCG2 antibody for two clones of each of the leucine mutants (25 μg of protein per lane), in most cases revealing significantly lower levels than in the R482G clone, from which they were derived. (B) Protein levels of the alanine mutants (15 μg of protein per lane). (C) RNA levels of one representative clone of each of the leucine and alanine mutants on Northern blot with one of the highest levels in the G406L/G410L mutant. Clone numbers are given above each panel.

Stable transfectants were generated in HEK293 cells by changing either or both glycines of the GXXXG motif at positions 406 and 410 to leucine or alanine using a pcDNA3.1 vector. Recently, it was observed in drug-resistant cell lines that mutation at amino acid 482, in the third transmembrane segment of ABCG2, confers differences in substrate specificity (*17*). In wild-type ABCG2 an arginine is encoded at this position, conferring narrower substrate specificity with resistance primarily to mitoxantrone and the camptothecins. A threonine or glycine encoded at amino acid 482 enables the protein to transport anthracyclines and rhodamine 123. In the present study we used this R482G "gain-of-function" mutation in all of the mutants in order to be able to study them with flow cytometry using rhodamine 123 as a substrate. Stable transfectants were obtained by selection in 2 mg/mL G418, and surviving clones were screened by flow cytometry using the 5D3 antibody, which detects ABCG2 on the cell surface. The clones positive for ABCG2 were expanded for further study.

Immunoblot Analysis of GXXXG Mutant Clones. Results of immunoblot analysis are shown with the anti-ABCG2 monoclonal antibody BXP21 for representative glycine and alanine mutant clones in panels A and B of Figure 2, respectively. ABCG2 runs as a 72 kDa protein on SDS−PAGE. When compared to the R482G transfectant clone, lower levels in most of the leucine mutants were consistently observed by immunoblot analysis. This was particularly true for the G406L/G410L double mutants. Figure 2C shows the RNA levels for one representative clone of each of the leucine and alanine mutants. Interestingly enough, the double leucine mutant had one of the highest levels of RNA on Northern blot, while representing the lowest level of protein on immunoblot.

Functional Assays. Since the lower levels observed by immunoblot analysis suggested that abnormalities in protein folding and/or dimerization could be present in these mutants, flow cytometry was performed to determine whether the mutant proteins had reached the cell surface and whether the proteins were functional. FACS analysis revealed all mutant proteins on the cell surface, using the 5D3 antibody, which was used to stain intact cells (Figure 3). To analyze the transport capacity of the GXXXG mutants, efflux of the following substrates was determined in the presence or absence of the ABCG2 inhibitor fumitremorgin C (FTC): rhodamine 123, mitoxantrone, pheophorbide *a*, and BODIPY-prazosin. Rhodamine 123 is transported by ABCG2 only when the R482G amino acid substitution is present. Evaluation of rhodamine 123 accumulation revealed almost complete abrogation of efflux in the clones carrying the glycine to leucine mutations. Assessment of mitoxantrone accumulation revealed some transport in the same mutants, but activity was impaired when compared to the R482G transfected clones. As indicated by almost no change in the level of fluorescence for mitoxantrone in the G406L/G410L mutant, this mutation impairs mitoxantrone transport more than the single mutations. Pheophorbide *a* is a dietary chlorophyll catabolite that has been demonstrated to induce photosensitivity in Abcg2 knockout mice (*39*). As shown in Figure 3 the double leucine mutant does not transport this compound, while the G406L and G410L mutants show impaired transport of pheophorbide *a*. Similar results were obtained with BODIPY-prazosin, a fluorescent conjugate of prazosin (Figure 3).

Transport studies for the GXXXG alanine mutants using the same ABCG2 substrates were also performed; Figure 3 shows the results obtained with the G406A/G410A mutant. Changes in fluorescence when FTC was added were almost identical to the R482G mutant, indicating that alanine is able to replace glycine without interfering with protein function. Flow cytometry also proved that the G406A and G410A mutants had intact transport of these substrates (data not shown). This result is not unexpected, since GXXXA, AXXXG, and AXXXA motifs are all known to occur at a higher than expected frequency in α-helices. The GXXXA and AXXXA motifs have both been implicated in stabilizing protein structures (*40*).

Cross-Linking Studies. Since substrate transport was impaired in the clones carrying the GXXXG glycine to leucine mutations, we evaluated the ability of the mutant proteins to dimerize, based on cross-linking studies, which serve as an indirect method for assessing dimerization. Using DSS, which cross-links proteins 11.4 Å apart, a dimer or higher order multimer was observed by immunoblot analysis in the G406L and G406L/G410L mutants and in the R482G control cells, as shown in Figure 4A. Next, cross-linking studies were performed with DSP, an agent that can be cleaved with the addition of DTT or β-mercaptoethanol.

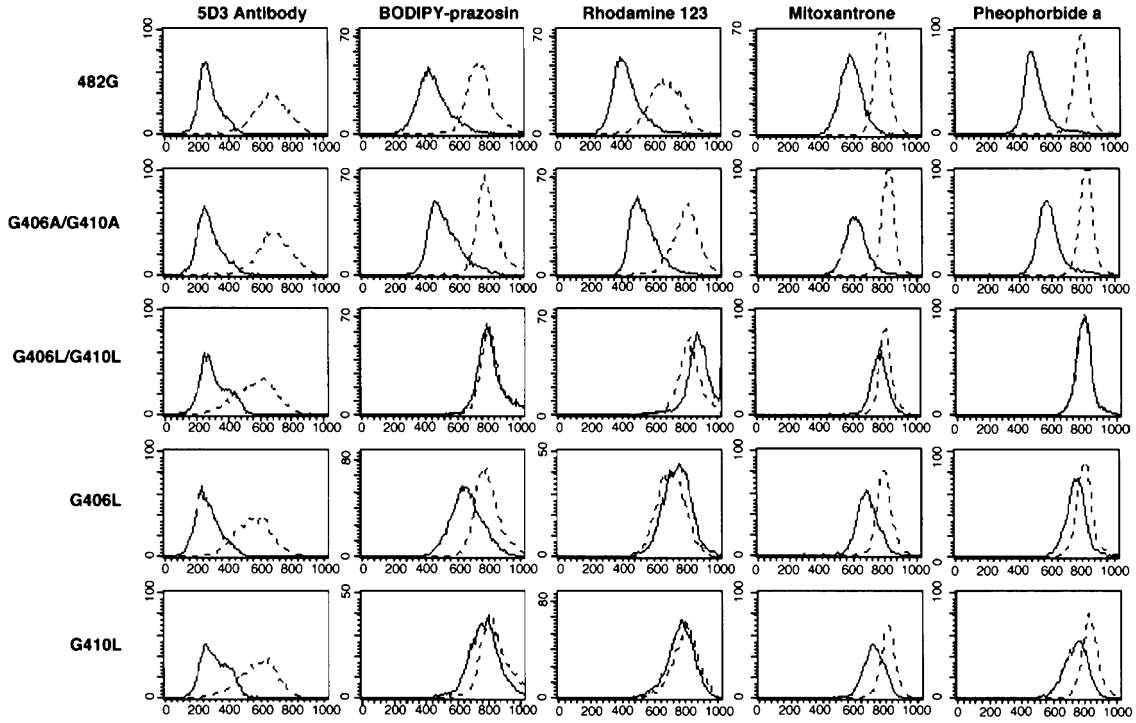

FIGURE 3: Flow cytometric analysis of transport capacity and cell surface expression of the GXXXG mutants. Using the 5D3 surface antibody all leucine and alanine mutant proteins are detectable on the cell surface by flow cytometry. Levels are compared to the R482G transfected cells. Plots: ABCG2 (---); negative control (—). Transport capacity for BODIPY-prazosin, rhodamine 123, mitoxantrone, and pheohorbide A in the R482G, G406A/G410A, G406L/G410L, G406L, and G410L transfected cells. Plots: Accumulation without FTC (—) and with FTC (---). Note lost BODIPY-prazosin and pheophorbide *a* transport with greatly impaired mitoxantrone transport in the double leucine mutant (G406L/G410L) and almost completely lost rhodamine 123 transport in all leucine mutants. The single leucine mutants (G406L, G410L) show impaired transport for BODIPY-prazosin, mitoxantrone, and pheophorbide *a* when compared to the fully functional R482G control cell line. The G406A/G410A mutants show intact transport capacity for all studied substrates.

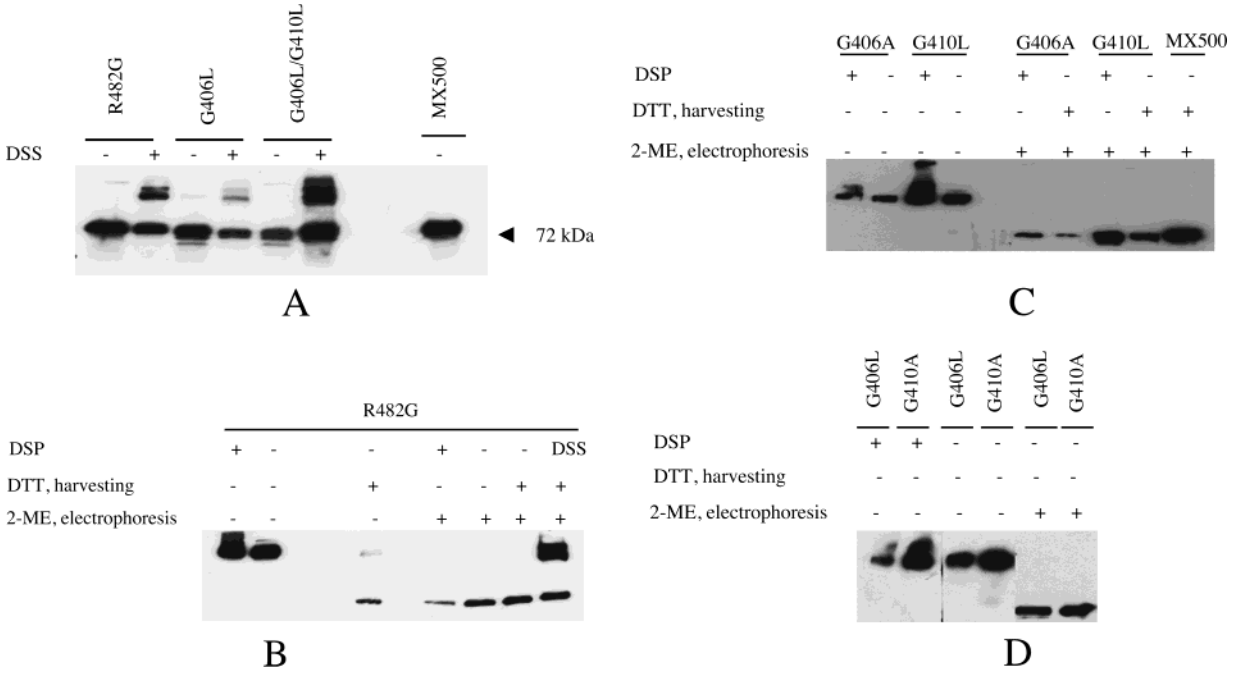

FIGURE 4: Cross-linking studies and nonreducing conditions. (A) Cross-linking of ABCG2 is observed with DSS in HEK293 cells transfected with R482G or with the functionally impaired leucine mutants. (B) Cross-linking is observed with the cleavable cross-linker DSP in the R482G mutant; note that the same higher molecular weight band can be seen under nonreducing conditions and that it can be reduced to the monomeric 72 kDa band with the addition of β-ME or DTT. (C) Dimerization is suggested in both G406A and G410L mutants by high molecular weight bands detected following either exposure to DSP or harvesting under nonreducing conditions. In either case, the addition of reducing agents generates a monomeric ABCG2 protein. (D) Similar results were obtained for the G406L and G410A mutants. (MX500 is a mitoxantrone-selected cell line used as a positive control.)

Membrane proteins were isolated from cells exposed to 2 mM DSP for 30 min and harvested with or without DTT in the lysis buffer. Running buffer with or without β-mercaptoethanol was used for SDS−PAGE gel electrophoresis.

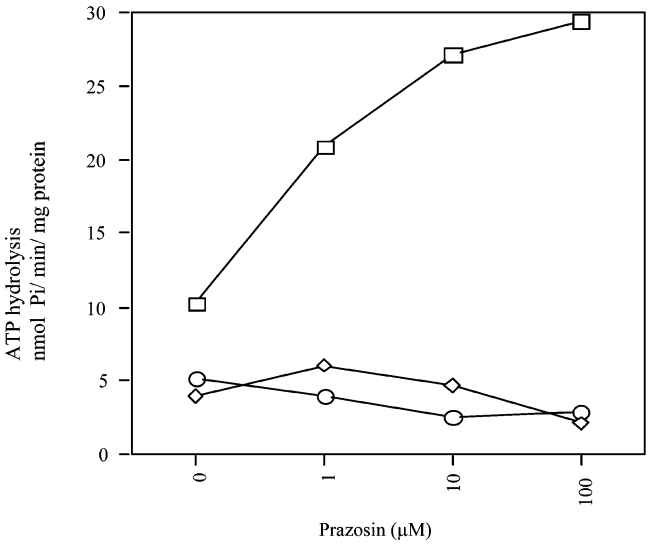

FIGURE 5: Substrate-stimulated ATP hydrolysis. The vanadate-sensitive ATP hydrolysis in the presence of the indicated concentrations of prazosin for crude membranes of HEK293 cells expressing ABCG2 R482G (□), G406L/G410L mutants (◇), and empty vector transfected cells (pcDNA; ○) is shown. The G406L/G410L mutant demonstrates basal ATPase levels identical to those of the pcDNA with no substrate induction observed. Each line represents the average of at least three measurements with at least two membrane preparations.

Cross-linking of the R482G transfected cells with DSP resulted in a high molecular weight protein, representing a dimer or multimer, which could be reduced to the monomeric 72 kDa form by exposure to reducing agents (Figure 4B). In addition, as previously reported by Kage et al. (*19*), proteins electrophoresed under nonreducing conditions also demonstrated high molecular weight species. Identical results were obtained with all the leucine and alanine mutants (Figure 4C showing results for the G406A and G410L mutants and Figure 4D for G406L and G410A; similar data not shown for the G406L/G410L and G406A/G410A mutants), implying that even if dimerization is impaired in these mutants, it has not prevented their close association on the cell surface.

ATPase Activity. Given that the results described thus far demonstrated a functional impact of TM1 mutation but no direct evidence of impaired dimerization, we analyzed the leucine mutant's ability to hydrolyze ATP. ABC transporters have previously been shown to exhibit drug-stimulated, vanadate-sensitive ATPase activity (*41*). For example, P-glycoprotein has been shown to have a 3−6-fold increase over baseline in ATPase activity in the presence of substrates. We determined the vanadate-sensitive component of the ATPase activity of the G406L/G410L mutant in the presence of 0, 1, 10, and 100 μM prazosin and compared it to the R482G and empty pcDNA3.1 vector transfected cells. Figure 5 shows that 100 μM prazosin increased the ATP hydrolysis activity of the R482G transfected clone approximately 3-fold over basal level, while the empty vector transfected control cell line (pcDNA) had a significantly lower basal level with a negligible increase in activity in the presence of the drug. Significantly, the G406L/G410L mutant, which previously showed the greatest impairment in transport capacity by flow cytometry (Figure 3), revealed ATPase levels identical to the empty vector transfected cell line with no stimulation with prazosin.

Treatment with Mitoxantrone. Previous studies by Loo and Clark with P-glycoprotein mutants suggest that addition of a transport substrate can improve folding and processing of a mutated ABC transporter (*42*). Since we observed reproducibly lower levels of the leucine mutants on immunoblot (Figure 2A), we considered the possibility that incubation of these cell lines in mitoxantrone could result in increased protein levels. Cells were thus incubated in 2−10 μM mitoxantrone overnight, membranes harvested, and proteins separated by electrophoresis. Figure 6A shows a significant increase in protein levels on imunoblot for the GXXXG motif mutants, with a less pronounced increase in the cells expressing protein without this mutation (R482G), reaching a plateau at 5 μM mitoxantrone. The increased ABCG2 expression after overnight treatment with mitoxantrone in the G406L and G406L/G410L mutants was also confirmed with confocal microscopy (Figure 6B). These results suggest that the ABCG2 substrate mitoxantrone may be able to improve protein stability.

DISCUSSION

As a half-transporter, ABCG2 is thought to homodimerize for function. In the present paper we report studies with mutants of ABCG2, evaluating residues that potentially contribute to the dimerization process. Glycine residues were substituted with leucine or alanine in a putative dimerization motif, GXXXG, found in the first TM of the ABCG2 protein. We isolated stable transfectants and studied protein expression and function of the mutants. While the double leucine mutant G406L/G410L was found on the cell surface, protein levels were reduced on immunoblot, and transport function and ATP hydrolysis were markedly impaired.

The GXXXG motif is the most frequently occurring sequence motif in transmembrane α-helices that has been implicated in the dimerization of several membrane proteins, including glycophorin A (*27*), human carbonic anhydrase (*28*), Ff bacteriophage major coat protein (*29*), yeast ATP synthase (*30*), *H. pylori* vacuolating toxin (*31*), yeast α-factor receptor (*32*), and members of the epidermal growth factor receptor (ErbB) family (*33*). In glycophorin A, homodimerization of the transmembrane segments is mediated by an extended sequence containing seven critical residues (LIXXGVXXGVXXT) (*43*) that create the interface between two right-handed α-helices. Typically accompanied by β-branched amino acids, the GXXXG region is thought to provide interacting grooves for α-helical connection. The four-residue separation aligns the glycines on one face of the helix, the lack of side chains in glycines allows the two helices to come into close proximity, and the dimer is thought to be stabilized by van der Waals interactions. Mutation of either of the glycines generally results in destabilization of dimerization (*44*).

The GXXXG motif has been identified experimentally as a dimerization motif by several separate strategies. One of these strategies was a rigorous analysis of sequence patterns in transmembrane segments, identifying GXXXG as the most highly biased sequence motif in naturally occurring transmembrane helices, occurring over 30% more frequently than expected (*26*). Another strategy used a 19 amino acid TM backbone of either leucines or alanines with random substitutions of the nine most commonly occurring TM residues

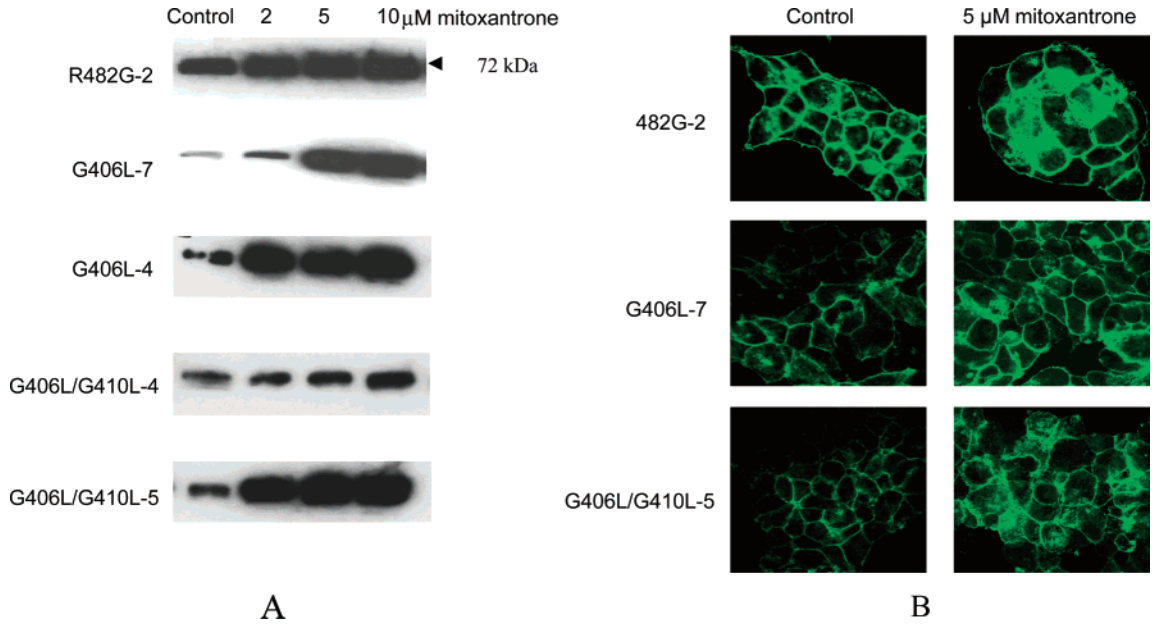

FIGURE 6: Impact of treatment with mitoxantrone on the levels of mutant ABCG2. (A) Overnight treatment of intact cells with 2−10 μM mitoxantrone substantially increases the level of ABCG2 in the GXXXG single and double leucine mutants (two clones for each shown) on immunoblot, while the change is much less pronounced in the control R482G transfected cells. (B) Confocal microscopy of HEK293 cells transfected with ABCG2 mutants shows a significant increase in ABCG2 levels for the G406L and G406L/G410L mutants after overnight treatment with 5 μM mitoxantrone. (The experiments shown in parts A and B were not performed simultaneously.)

at positions 1, 2, 5, 6, 9, 10, and 13 (*25*). The resulting TMs were inserted into a TOXCAT vector (*45*). This vector generates a hybrid protein between the TM construct and a dimerization-dependent transcriptional activator. The dimerized transcription factor activates chloramphenicol acetyltransferase, which confers chloramphenicol resistance. Selection of colonies in chloramphenicol allows isolation of vectors containing TMs that are able to dimerize. The most frequently occurring TM dimer in this model system contained glycines at positions 5 and 9; 96% of the leucine-based TM backbones had glycines in these positions, forming a GXXXG motif. In most cases when glycine was not present in these positions, it was replaced by serine, which, like glycine and alanine, is one the smallest amino acids and therefore can permit close proximity of TMs.

The GXXXG motif in TM1 is highly conserved in the ABCG family, with ABCG1, ABCG2, and ABCG4 each containing the motif. Immunoblot, flow cytometry, and ATP hydrolysis assays presented in this paper have shown that replacement of the glycines with leucines resulted in a protein with impaired function. Flow cytometric analysis of the GXXXG leucine mutants showed impaired or lost transport in the case of all studied substrates. On the other hand, replacement with alanines yielded a fully functional protein. Treating cells with cross-linking agents prior to protein isolation demonstrated a dimer or higher order multimer in the mutants containing either leucine or alanine at the GXXXG site, as well as in the wild-type cells. These results demonstrated that while the GXXXG motif in TM1 is important for proper function and might be involved in dimerization, it cannot alone be responsible for ABCG2 dimerization. These findings suggest that other residues or other proteins must be involved in the dimerization process. Indeed, as previously mentioned, the most extensively studied glycophorin A dimerization motif consists of at least seven residues (*43*). A flanking threonine has been found to be especially important in glycophorin A dimerization at position 13, relative to the glycines at positions 5 and 9.

In addition to the possibility that the GXXXG motif is surrounded by other residues required for dimerization, TM1 could also interact with one of the other five TM segments in the ABCG2 molecule. AXXXA, a motif that is also known to be involved in dimerization of α-helices, is found in TM4 (*38*). This possibility is not addressed by the cross-linking studies, which merely show ABCG2 molecules in close proximity. Analysis of the primary structure of the best studied ABC transporter, P-glycoprotein, reveals the presence of conserved A/S/GXXXA/S/G motifs in 9 out of 12 transmembrane helices. Since it is not plausible that all those helices would be involved in intermolecular interactions, it seems likely that some of the motifs provide for tight intramolecular packing between domains.

The results presented in this paper suggest abnormal protein processing and function when the glycines of the GXXXG motif in TM1 of ABCG2 are mutated to leucines. Although the mutant proteins are located on the cell surface, they are expressed at a lower level than the R482G mutant, to which they should be compared. Addition of mitoxantrone to the cells carrying the leucine mutants results in increased levels of ABCG2 detected by immunoblot analysis. These results are similar to those observed following incubation of cells expressing mutant P-glycoprotein with either substrates or inhibitors (*42*). Loo and Clark reported that the addition of vinblastine, capsaicin, cyclosporin, or verapamil to HEK293 cells transiently transfected with P-glycoprotein mutants improved protein processing. The increase in ABCG2 levels on immunoblot and on the cell surface following overnight treatment of the mutant cell lines with mitoxantrone is consistent with improved processing or alternatively protection against increased degradation. Although the ubiquitin−proteasome pathway is an important mechanism of protein degradation by which the cell is able to

maintain the integrity of protein structure, some misfolded proteins might end up as insoluble aggregates in cells (*46*). The fact that no increase in the level of ubiquitination was seen after treatment with mitoxantrone rules out the possibility that inhibition of this pathway by mitoxantrone would be accountable for the observed increase in mutant protein levels (data not shown).

The working paradigm is that ABC half-transporters must dimerize to form a functional transporter. The recently obtained crystal structure of MsbA, a bacterial half-transporter protein related to human P-glycoprotein, suggests that the dimerization interface in this type of ABC transporter is formed primarily by TM1 and TM6 of opposing monomers (*23*, *24*). In the closed position, the two helices contributed from each half interlock with the mirror image of the corresponding TM segments of the opposing monomer. BtuCD, the bacterial vitamin B_{12} transporter, is comprised of two TM domains and two ATP-binding domains. Its crystal structure reveals a similar dimer interface formed by packing of two pairs of opposing TM helices, creating a central channel (*47*). These structures suggest that, similarly, two pairs of helices may be involved in the ABCG2 dimer interface.

Remarkably, both the basal and prazosin-stimulated ATPase activity was found to be severely impaired in the G406L/G410L mutant. Considering P-glycoprotein as a model, basal ATPase activity has primarily been found to be affected by mutations in the ATP-binding sites. It is, however, clear from work in several laboratories that mutations in other regions, including transmembrane domains, can impair the substrate-stimulated ATP hydrolysis (*48*). Interestingly, it has been demonstrated that the deletion of residues 653−686 in the linker region of P-glycoprotein inhibits transport and impairs both the basal and the substrate-stimulated ATP hydrolysis, without changing the cell surface expression (*49*). These data suggested that the linker region of P-glycoprotein is critical for the proper alignment of the two ATP sites and might be involved in signaling between the substrate- and ATP-binding sites. Since both the basal and the prazosin-stimulated ATP hydrolyses were impaired in the G406L/G410L mutant, it is conceivable that the GXXXG motif could play a similar role in ABCG2.

CONCLUSION

The high degree of glycine residue conservation in the first TM of ABCG transporters, along with the described mutational data, strongly suggests that the GXXXG motif is, at the very least, essential for the protein assembly and is very likely to be involved in interaction with other TMs. The GXXXG motif of ABCG2 could be viewed as a structural element that facilitates dimerization by allowing packing of the helices but does not necessarily induce dimerization single-handedly. Clearly, these residues are important in ABCG2 function. Future studies are needed to determine the precise components involved in ABCG2 dimerization.

ACKNOWLEDGMENT

The authors thank Dr. Gary Kruh for helpful advice regarding transfection in the HEK 293 system, Dr. Yuan Ting for technical assistance with site-directed mutagenesis, Drs. Dan Sackett, Tito Fojo, and Marianne Poruchynsky for helpful advice in protein studies, Sara Runge and Michael Rapawy for performing some of the transfections and immunoblots included in this paper, and Yvona Ward, Csilla Ozvegy, and Laszlo Homolya for assistance with confocal microscopy.

REFERENCES

1. Gottesman, M. M., Fojo, T., and Bates, S. E. (2002) Multidrug resistance in cancer: role of ATP-dependent transporters, *Nat. Rev. Cancer 2*, 48−58.
2. Dean, M., Hamon, Y., and Chimini, G. (2001) The human ATP-binding cassette (ABC) transporter superfamily, *J. Lipid Res. 42*, 1007−1017.
3. Miyake, K., Mickley, L., Litman, T., Zhan, Z., Robey, R., Cristensen, B., Brangi, M., Greenberger, L., Dean, M., Fojo, T., and Bates S. E. (1999) Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: demonstration of homology to ABC transport genes, *Cancer Res. 59*, 8−13.
4. Doyle, L. A., Yang, W., Abruzzo, L. V., Krogmann, T., Gao, Y., Rishi, A. K., and Ross, D. D (1998) A multidrug resistance transporter from human MCF-7 breast cancer cells, *Proc. Natl. Acad. Sci. U.S.A. 95*, 15665−15670.
5. Allikmets, R., Schriml, L. M., Hutchinson, A., Romano-Spica, V., and Dean, M. (1998) A human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance, *Cancer Res. 58*, 5337−5339.
6. Nakagawa, M., Schneider, E., Dixon, K. H., Horton, J., Kelley, K., Morrow, C., and Cowan, K. H. (1992) Reduced intracellular drug accumulation in the absence of P-glycoprotein (mdr1) overexpression in mitoxantrone-resistant human MCF-7 breast cancer cells, *Cancer Res. 52*, 6175−6181.
7. Hazlehurst, L. A., Foley, N. E., Gleason-Guzman, M. C., Hacker, M. P., Cress, A. E., Greenberger, L. W., De Jong, M. C., and Dalton, W. S. (1999) Multiple mechanisms confer drug resistance to mitoxantrone in the human 8226 myeloma cell line, *Cancer Res. 59*, 1021−1028.
8. Robey, R. W., Medina-Perez, W. Y., Nishiyama, K., Lahusen, T., Miyake, K., Litman, T., Senderowicz, A. M., Ross, D. D., and Bates, S. E. (2001) Overexpression of the ATP-binding cassette half-transporter, ABCG2 (Mxr/BCrp/ABCP1), in flavopiridol-resistant human breast cancer cells, *Clin. Cancer Res. 7*, 145−152.
9. Chen, Z. S., Robey, R. W., Belinsky, M. G., Shchaveleva, I., Ren, X. Q., Sugimoto, Y., Ross, D. D., Bates, S. E., and Kruh, G. D. (2003) Transport of methotrexate, methotrexate polyglutamates, and 17beta-estradiol 17-(beta-D-glucuronide) by ABCG2: effects of acquired mutations at R482 on methotrexate transport, *Cancer Res. 63*, 4048−4054.
10. Kawabata, S., Oka, M., Shiozawa, K., Tsukamoto, K., Nakatomi, K., Soda, H., Fukuda, M., Ikegami, Y., Sugahara, K., Yamada, Y., Kamihira, S., Doyle, L. A., Ross, D. D., and Kohno, S. (2001) Breast cancer resistance protein directly confers SN-38 resistance of lung cancer cells, *Biochem. Biophys. Res. Commun. 280*, 1216−1223.
11. Maliepaard, M., van Gastelen, M. A., de Jong, L. A., Pluim, D., van Waardenburg, R. C., Ruevekamp-Helmers, M. C., Floot, B. G., and Schellens, J. H. (1999) Overexpression of the BCRP/MXR/ABCP gene in a topotecan-selected ovarian tumor cell line, *Cancer Res. 59*, 4559−4563.
12. Berkower, C., and Michaelis, S. (1991) Mutational analysis of the yeast a-factor transporter STE6, a member of the ATP binding cassette (ABC) protein superfamily, *EMBO J. 10*, 3777−3785.
13. Chen, C. J., Chin, J. E., Ueda, K., Clark, D. P., Pastan, I., Gottesman, M. M., and Roninson, I. B. (1986) Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells, *Cell 47*, 381−389.
14. Kaur, P., and Russell, J. (1998) Biochemical coupling between the DrrA and DrrB proteins of the doxorubicin efflux pump of Streptomyces peucetius, *J. Biol. Chem. 273*, 17933−17939.
15. Graf, G. A., Li, W. P., Gerard, R. D., Gelissen, I., White, A., Cohen, J. C., and Hobbs, H. H. (2002) Coexpression of ATP-binding cassette proteins ABCG5 and ABCG8 permits their transport to the apical surface, *J. Clin. Invest. 110*, 659−669.
16. Allen, J. D., Van Dort, S. C., Buitelaar, M., van Tellingen, O., and Schinkel, A. H. (2003) Mouse breast cancer resistance protein

(Bcrp1/Abcg2) mediates etoposide resistance and transport, but etoposide oral availability is limited primarily by P-glycoprotein, *Cancer Res. 63*, 1339−1344.
17. Honjo, Y., Hrycyna, C. A., Yan, Q. W., Medina-Perez, W. Y., Robey, R. W., van de Laar, A., Litman, T., Dean, M., and Bates, S. E. (2001) Acquired mutations in the MXR/BCRP/ABCP gene alter substrate specificity in MXR/BCRP/ABCP-overexpressing cells, *Cancer Res. 61*, 6635−6639.
18. Özvegy, C., Litman, T., Szakacs, G., Nagy, Z., Bates, S., Varadi, A., and Sarkadi, B. (2001) Functional characterization of the human multidrug transporter, ABCG2, expressed in insect cells, *Biochem. Biophys. Res. Commun. 285*, 111−117.
19. Kage, K., Tsukahara, S., Sugiyama, T., Asada, S., Ishikawa, E., Tsuruo, T., and Sugimoto, Y. (2002) Dominant-negative inhibition of breast cancer resistance protein as drug efflux pump through the inhibition of S−S dependent homodimerization, *Int. J. Cancer 97*, 626−630.
20. Poelarends, G. J., and Konings, W. N. (2002) The transmembrane domains of the ABC multidrug transporter LmrA form a cytoplasmic exposed, aqueous chamber within the membrane, *J. Biol. Chem. 277*, 42891−42898.
21. van Veen, H. W., Venema, K., Bolhuis, H., Oussenko, I., Kok, J., Poolman, B., Driessen, A. J., and Konings, W. N.(1996) Multidrug resistance mediated by a bacterial homolog of the human multidrug transporter MDR1, *Proc. Natl. Acad. Sci. U.S.A. 93*, 10668−10672.
22. Ewart, G. D., Cannell, D., Cox, G. B., and Howells, A. J. (1994) Mutational analysis of the traffic ATPase (ABC) transporters involved in uptake of eye pigment precursors in Drosophila melanogaster. Implications for structure−function relationships, *J. Biol. Chem. 269*, 10370−10377.
23. Shilling, R. A., Balakrishnan, L., Shahi S., Venter H., and van Veen, H. W. (2003) A new dimer interface for an ABC transporter, *Int. J. Antimicrob. Agents 22*, 200−204.
24. Chang, G., and Roth, C. B. (2001) Structure of MsbA from *E. coli*: a homolog of the multidrug resistance ATP binding cassette (ABC) transporters, *Science 293*, 1793−1800.
25. Senes, A., Gerstein, M., and Engelman, D. M. (2000) Structure of MsbA from *Vibrio cholera*: a multidrug resistance ABC transporter homolog in a closed conformation, *J. Mol. Biol. 296*, 921−936.
26. Russ, W. P., and Engelman, D. M. (2000) The GxxxG motif: a framework for transmembrane helix-helix association, *J. Mol. Biol. 296*, 911−919.
27. Gerber, D., and Shai, Y. (2001) In vivo detection of hetero-association of glycophorin-A and its mutants within the membrane, *J. Biol. Chem. 276*, 31229−31232.
28. Whittington, A., Waheed, A., Ulmasov, B., Shah, G. N., Grubb, J. H., Sly, W. S., and Christianson, D. W. (2001) In vivo detection of hetero-association of glycophorin-A and its mutants within the membrane, *Proc. Natl. Acad. Sci. U.S.A. 98*, 9545−9550.
29. Melnyk, R. A., Partridge, A. W., and Deber, C. M. (2002) Transmembrane domain mediated self-assembly of major coat protein subunits from Ff bacteriophage, *J. Mol. Biol. 315*, 63−72.
30. Arselin, G., Giraud, M. F., Dautant, A., Vaillier, J., Brethes, D., Coulary-Salin, B., Schaeffer, J., and Velours, J. (2003) The GxxxG motif of the transmembrane domain of subunit e is involved in the dimerization/oligomerization of the yeast ATP synthase complex in the mitochondrial membrane, *Eur. J. Biochem. 270*, 1875−1884.
31. McClain, M. S., Iwamoto, H., Cao, P., Vinion-Dubiel, A. D., Li, Y., Szabo, G., Shao, Z., and Cover, T. L. (2003) Essential role of a GXXXG motif for membrane channel formation by *Helicobacter pylori* vacuolating toxin, *J. Biol. Chem. 278*, 12101−12108.
32. Overton, M. C., Chinault, S. L., and Blumer, K. J. (2003) Oligomerization, biogenesis, and signaling is promoted by a glycophorin A-like dimerization motif in transmembrane domain 1 of a yeast G protein-coupled receptor, *J. Biol. Chem. 278*, 49369−49377.
33. Mendrola, J. M., Berger, M. B., King, M. C., and Lemmon, M. A. (2002) The single transmembrane domains of ErbB receptors self-associate in cell membranes, *J. Biol. Chem. 277*, 4704−4712; Cover, T. L. (2003) *J. Biol. Chem. 278*, 12101−12108.
34. Litman, T., Jensen, U., Hansen, A., Covitz, K. M., Zhan, Z., Fetsch, P., Abati, A., Hansen, P. R., Horn, T., Skovsgaard, T., and Bates, S. E. (2002) Use of peptide antibodies to probe for the mitoxantrone resistance-associated protein MXR/BCRP/ABCP/ABCG2, *Biochim. Biophys. Acta 1565*, 6−16.
35. Ozvegy, C. Cs., Varadi, A., and Sarkadi (2002) Characterization of drug transport, ATP hydrolysis, and nucleotide trapping by the human ABCG2 multidrug transporter. Modulation of substrate specificity by a point mutation, *J. Biol. Chem. 277*, 47980−47990.
36. Hrycyna, C. A., Ramachandra, M., Pastan, I., and Gottesman, M. M. (1998) Functional expression of human P-glycoprotein from plasmids using vaccinia virus-bacteriophage T7 RNA polymerase system, *Methods Enzymol. 292*, 456−473.
37. Liu, Y., Engelman, D. M., and Gerstein, M. (2002) Genomic analysis of membrane protein families: abundance and conserved motifs, *Genome Biol. 3*, 1−12.
38. Kleiger, G., Grothe, R., Mallick, P., and Eisenberg, D. (2002) GXXXG and AXXXA: common alpha-helical interaction motifs in proteins, particularly in extremophiles, *Biochemistry 41*, 5990−5997.
39. Jonker, J. W., Buitelaar, M., Wagenaar, E., Van Der Valk, M. A., Scheffer, G. L., Scheper, R. J., Plosch, T., Kuipers, F., Elferink, R. P., Rosing, H., Beijnen, J. H., and Schinkel, A. H. (2002) The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria, *Proc. Natl. Acad. Sci. U.S.A. 99*, 15649−15654.
40. Kleiger, G., and Eisenberg, D. (2002) GXXXG and GXXXA motifs stabilize FAD and NAD(P)-binding Rossmann folds through C(alpha)−H···O hydrogen bonds and van der Waals interactions, *J. Mol. Biol. 323*, 69−76.
41. Ambudkar, S. V., Lelong, I. H., Zhang, J., Cardarelli, C. O., Gottesman, M. M., and Pastan, I. (1992) Partial purification and reconstitution of the human multidrug-resistance pump: characterization of the drug-stimulatable ATP hydrolysis, *Proc. Natl. Acad. Sci. U.S.A. 89*, 8472−8476.
42. Loo, T. W., and Clarke, D. M. (1997) Correction of defective protein kinesis of human P-glycoprotein mutants by substrates and modulators, *J. Biol. Chem. 272*, 709−712.
43. Brosig, B., and Langosch, D. (1998) The dimerization motif of the glycophorin A transmembrane segment in membranes: importance of glycine residues, *Protein Sci. 7*, 1052−1056.
44. Lemmon, M. A., Flanagan, J. M., Treutlein, H. R., Zhang, J., and Engelman, D. M. (1992) Sequence specificity in the dimerization of transmembrane alpha-helices, *Biochemistry 31*, 12719−12725.
45. Russ, W. P., and Engelman, D. M. (1999) TOXCAT: a measure of transmembrane helix association in a biological membrane, *Proc. Natl. Acad. Sci. U.S.A. 96*, 863−868.
46. Johnston, J. A., Ward, C. L., and Kopito, R. R. (1998) Aggresomes: a cellular response to misfolded proteins, *J. Cell Biol. 143*, 1883−1898.
47. Locher, K. P., and Borths, E. (2004) ABC transporter architecture and mechanism: implications from the crystal structures of BtuCD and BtuF, *FEBS Lett. 564*, 264−268.
48. Ambudkar, S. V., Dey, S., Hrycyna, C. A., Ramachandra, M., Pastan, I., and Gottesman, M. M. (1999) Biochemical, cellular, and pharmacological aspects of the multidrug transporter, *Annu. Rev. Pharmacol. Toxicol. 39*, 361−398.
49. Hrycyna, C. A., Airan, L. E., Germann, U. A., Ambudkar, S. V., Pastan, I., and Gottesman, M. M. (1998) Structural flexibility of the linker region of human P-glycoprotein permits ATP hydrolysis and drug transport, *Biochemistry 37*, 13660−13673.

BI0497953