Effect of Walker A mutation (K86M) on oligomerization and surface targeting of the multidrug resistance transporter ABCG2

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Summary

The ATP binding cassette (ABC) half-transporter ABCG2 (MXR/BCRP/ABCP) is associated with mitoxantrone resistance accompanied by cross-resistance to a broad spectrum of cytotoxic drugs. Here we investigate the functional consequences of mutating a highly conserved lysine in the Walker A motif of the nucleotide binding domain (NBD) known to be critical for ATP binding and/or hydrolysis in ABC transporters. The mutant (ABCG2-K86M) was inactive as expected but was expressed at similar levels as the wild-type (wt) protein. The mutation did not affect the predicted oligomerization properties of the transporter; hence, co-immunoprecipitation experiments using differentially tagged transporters showed evidence for oligomerization of both ABCG2-wt and of ABCG2-wt with ABCG2-K86M. We also obtained evidence that both ABCG2-wt and ABCG2-K86M exist in the cells as disulfide-linked dimers. Moreover, measurement of prazosin-stimulated ATPase activity revealed a dominant-negative effect of ABCG2-K86M on ABCG2-wt function in co-transfected HEK293 cells. This is consistent with the requirement for at least two active NBDs for transporter activity and suggests that the transporter is a functional dimer. Finally, we analyzed targeting of ABCG2-wt and ABCG2-K86M and observed that they localize to two distinct subcellular compartments: ABCG2-wt targets the cell surface whereas ABCG2-K86M is targeted to the Golgi apparatus followed by retrieval to the endoplasmic reticulum. This suggests an as yet unknown role of the NBDs in assisting proper surface targeting of ABC transporters.

Key words: ABC transporters, ABCG2, Oligomerization, Trafficking, Multidrug resistance, Walker A mutation

Introduction

Multidrug resistance (MDR) represents a serious problem in cancer chemotherapy. The resistant tumor cells often overexpress one of several ATP binding cassette (ABC) transporters that are capable of mediating efflux of many clinically important drugs (Litman et al., 2001). These transporters include among others P-glycoprotein, the multidrug resistance associated protein 1 (MRP1) and ABCG2. ABCG2 is expressed in many different cancer tissues (Diestra et al., 2002) and several different types of leukemia (Ross et al., 2000; Sauerbrey et al., 2002). ABCG2 expression is upregulated particularly in cells exposed to mitoxantrone, a drug often used in the treatment of breast cancer (Diah et al., 2001). ABCG2 is situated in the plasma membrane (Rocchi et al., 2000) where it mediates efflux not only of mitoxantrone, but also of flavopiridol, camptothecins and methotrexate (Brangi et al., 1999; Litman et al., 2000; Robey et al., 2001; Volk et al., 2002). It should be noted that an amino acid substitution at position 482 distinguishes MXR (R482G), BCRP (R482T) and ABCP (R482, wt) (Allikmets et al., 1998; Doyle et al., 1998; Miyake et al., 1999), which are synonymous designations for ABCG2. This substitution has been shown to influence the substrate specificity of the transporter (Chen et al., 2003b; Mitomo et al., 2003; Ozvegy et al., 2002; Robey et al., 2003).

In normal tissue, ABCG2 has the highest expression level in the placenta and is also expressed in many other tissues such as the ducts and lobules of the breast, in the small and large intestine and the canalicular membrane of the liver (Maliepaard et al., 2001). Although a physiological function of ABCG2 remains to be established, the tissue distribution might suggest a role of the protein in protection against xenobiotics. Interestingly, ABCG2 has been associated with the side population phenotype of hematopoietic stem cells (Kim et al., 2002; Zhou et al., 2001); however, the function of ABCG2 in stem cells is still unclear. Finally, ABCG2 has been implicated in the transport of sterols, as have several of the ABCG-subfamily members (Janvilisri et al., 2003).

ABC transporters are expressed as either full transporters with two nucleotide binding domains (NBDs) and two transmembrane domains (TMDs) in one polypeptide, or as half-transporters with one NBD and one TMD. ABCG2 is an ABC half-transporter consisting of an N-terminal NBD and a C-terminal TMD with six putative α-helical transmembrane
segments. For other half-transporters strong experimental evidence suggests that they dimerize and form functional homo- or heterodimers (Graf et al., 2002; Liu et al., 1999; van Veen et al., 2000). ABCG2 is also thought to function as a homodimer (Kage et al., 2002; Litman et al., 2002; Ozvegy et al., 2002); nonetheless, the requirement for two functional NBDs for the ATP-dependent transport activity of ABCG2 has not been experimentally tested.

In the present study we have investigated the effect of mutating a conserved lysine in Walker A of ABCG2. This lysine is known from both mammalian and bacterial ABC transporters to be critical for ATP binding and/or hydrolysis (Davidson and Sharma, 1997; Lapinski et al., 2001; Muller et al., 1996; Ozvegy et al., 2002; Szabo et al., 1998; van Veen et al., 2000). In ABCG2, ATP binds but is not hydrolyzed (Ozvegy et al., 2002). In agreement, we find that the mutant (ABCG2-K86M) is inactive. The predicted oligomerization is, however, not affected and we observe a dominant-negative effect on ATPase activity. Finally, we observe that the mutation not only inactivates the transporter but also alters the subcellular distribution of the transporter.

Materials and Methods

Plasmids, drugs and antibodies
pcDNA3.1(−)MXR and Fumitremorgin C (FTC) were kindly provided by Susan Bates, National Cancer Institute, NIH. BODIPY-prazosin was obtained from Molecular Probes (Eugene, OR). All other chemicals were obtained from Sigma (St Louis, MO). Antibodies against the following were used: ABCG2, ABCG2-PE and IgG-PE (eBioscience, San Diego, CA), BXP-21 (Alexis Antibodies) or 1:50,000 (α-actin, β-actin) were developed using Supersignal chemiluminescent substrate (Pierce, Rockford, IL). For the reduction analysis the samples were incubated with or without 100 mM DTT for 30 minutes. 10 mM DTT was added to the loading buffer.

Construction of mutation and tags
The ABCG2-K86M mutation was generated by a two-generation PCR technique using the Pfu polymerase (Stratagene, La Jolla, CA). The PCR fragments were digested with the appropriate enzymes, purified by agarose gel electrophoresis and cloned into either pCIN4 (confers G418 resistance) or pciHygro (confers hygromycin resistance) (Rees et al., 1996). ABCG2-wt was tagged with the MYC epitope whereas ABCG2-ABCG2-wt tagged with the HA epitope whereas ABCG2-ABCG2-wt tagged with the HA epitope. These constructs were also generated by PCR-derived mutagenesis and cloned into pCIN4 or pciHygro. All constructs were confirmed by restriction enzyme mapping and DNA sequencing using an ABI 310 automated sequencer according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA).

Cell culture and stable transfection
HEK293 cells were maintained at 37°C, 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) with Glutamax I supplemented with 10% fetal calf serum and 0.01 mg/ml gentamicin (all products from Invitrogen, Carlsbad, CA). For stable transfections, the HEK293 cells were seeded in 10 µg/ml poly-D-lysine coated 75 cm² flasks 1 day before transfection. Cells were transfected with 5 µg pCIN4 or pciHygro constructs using the Lipofectamine™/Opti-MEM™ transfection system. A stably transfected pool clone was selected using Geneticin (G418) (0.35 mg/ml) or hygromycin (0.35 mg/ml), respectively. For stable co-transfections, a G418-resistant cell line expressing ABCG2-wt-MYC was transfected with the appropriate pciHygro construct and stable pool clones expressing both constructs were selected by combined selection with G418 (0.2 mg/ml) and hygromycin (0.2 mg/ml).

Preparation of membrane fractions
HEK293 microsomal membranes were prepared as previously described (Litman et al., 1997). The membrane pellets were resuspended in lysis buffer (10 mM HEPES–Tris-HCl, pH 7.4, 5 mM EDTA, 5 mM EGTA, 2 mM PMSF, 2 mM DTT, protease inhibitor cocktail), homogenized by aspiration with a fine needle (28 gauge), aliquoted and stored at −80°C until further analysis.

Western blotting
Protein samples from various experiments were added to 2× SDS loading buffer (+100 mM DTT) and incubated for 30 minutes at 37°C. The samples were analyzed by SDS-PAGE, blotted onto a PVDF or nitrocellulose membrane and incubated in TST blocking buffer (10 mM Tris-HCl, 2.5 mM EDTA, 100 mM NaCl and 0.1% Tween 20 with 5% milk). Primary antibodies were diluted 1:1000 (HA, Ab-405, BXP-21, Ab-405) or 1:50,000 (β-actin) and secondary antibodies 1:10,000 (goat-anti-rabbit or goat-anti-mouse). The blots were developed using Supersignal chemiluminescent substrate (Pierce, Rockford, IL). For the reduction analysis the samples were incubated with or without 100 mM DTT for 30 minutes. 10 mM N-ethylmaleimide (NEM) was added to the loading buffer.

Co-immunoprecipitation
Cells were grown to 90% confluence in six-well plates and harvested in solubilization buffer [150 mM NaCl, 25 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.2 mM PMSF, 5 mM NEM, 1% Triton X-100 and protease inhibitors] while kept on ice. After a 30 minute incubation with end-over-end rotation at 4°C, the lysates were cleared by centrifugation at 16,000 g for 30 minutes. Primary antibody (1:100 MYC) was added to 500 µg total protein per sample and the samples were incubated for 3 hours at 4°C followed by incubation with G-protein agarose for 3 hours at 4°C. After washing thoroughly, the precipitate was eluted by adding SDS loading buffer without DTT. Before analysis by western blotting, 100 mM DTT was added to the samples followed by incubation for 30 minutes at 37°C.

Immunocytochemistry
Cells were seeded on poly-D-lysine coated coverslips. After 24 hours the cells were fixed by adding 3.7% formaldehyde in cold PBS for 20 minutes at 4°C. After fixation, the cells were washed three times with PBS and incubated in blocking buffer (PBS, 5% goat serum, 0.1% Triton X-100 or 0.2% saponin) for 20 minutes at room temperature. The cells were incubated with primary antibody (1:500 anti-ABCG2, 1:500 anti-giantin or 1:200 anti-calnexin) in blocking buffer for 1 hour and washed. Then, the secondary antibody conjugated to a fluorescent probe (Alexa 488 or Alexa 568) was added at 1:200. Finally, the cells were washed three times in PBS before the coverslips were mounted with antifade (Molecular Probes, Eugene, OR) and analyzed by confocal microscopy (LSM510, Zeiss) or epifluorescence (Axiovert 200, Zeiss).

ATPase activity assay
The ATPase activity was determined according to a published method (Borgnia et al., 1996), using a colorimetric assay described (Chifflet et al., 1988). Each experiment was carried out in a 96-well microtiter plate (Nunc, Roskilde, Denmark) with reaction volumes of 50 µl/well.
corresponding to 5 µg protein/well. Incubation with prazosin was carried out at 37°C and the color was left to develop at room temperature for 30 minutes before phosphate release was quantified at 750 nm.

Cytotoxicity assay
The cytotoxicity assay was performed as described (Skehan et al., 1990). Stably transfected HEK293 cells were seeded (2000 cells/well) in sterile 96-well plates (Costar, Corning, Acton, MA) in 100 µl DMEM with Glutamax and incubated for 24 hours at 37°C. Mitoxantrone was added at ten different final concentrations in 100 µl DMEM. After incubation for 72 hours at 37°C, the cells were fixed in 50% TCA. The fixed cells were stained with 100 µl 0.4% sulforhodamine B (SRB) in 1% acetic acid. Finally, the SRB dye was dissolved in 10 mM unbuffered Tris base (pH 10.5) for 10 minutes and measured at OD595 in a microplate reader (Bio-Tek Instruments, Winooski, VT).

Efflux assay
The efflux assay was performed as described (Lee et al., 1994). A 175 cm² flask of stably transfected HEK293 cells was harvested and resuspended in 10 ml IMEM and washed once. During the accumulation period, three aliquots of cells were suspended in 100 µl IMEM containing DMSO, BODIPY-prazosin (100 nM) or BODIPY-prazosin (100 nM) and 5 µM FTC and incubated for 30 minutes at 37°C. After incubation the cells were centrifuged for 3 minutes at 1000 g. The background controls were resuspended in 1 ml ice-cold PBS and left on ice in the dark. The other samples were washed in PBS and resuspended in 1 ml IMEM with or without 5 µM FTC and incubated for 60 minutes at 37°C. Following incubation, the samples were treated as the background samples and analyzed by flow cytometry using a Becton Dickinson FACScalibur. The data were analyzed in Cell Quest Pro (BD Biosciences, San Jose, CA).

Surface staining
Stably transfected HEK293 cells were grown to 60% confluence. The cells were harvested and stained in IMEM and washed once (PBS, 5% FCS). PE-conjugated anti-ABCG2 (1:25) or IgG isotype control (1:25) were added to the cells and the samples were incubated for 30 minutes in the dark at room temperature. The cells were washed three times in staining buffer and finally resuspended in staining buffer. 20 µl Propidium Iodide was added to each sample to exclude dead cells during FACS analysis. Finally, the samples were analyzed by flow cytometry using FACScalibur and the data were analyzed by Cell Quest Pro.

Deglycosylation
Total cell lysates were prepared from stably transfected cells grown to 90% confluence in six-well plates and harvested in solubilization buffer (150 mM NaCl, 25 mM Tris, 1 mM EDTA, 0.2 mM PMSF, 5 mM NEM, 1% Triton X-100 and protease inhibitors) on ice. After a 30-minute incubation with end-over-end rotation at 4°C, the lysates were cleared by centrifugation at 16,000 g for 30 minutes. Samples were treated according to the manufacturer’s protocol (New England Biolabs, Beverly, MA). Briefly, 15 µg total protein/sample was incubated for 10 minutes at 100°C in denaturation buffer (5% SDS, 10% β-mercaptoethanol) before incubation with 100 µl PNGase F or endonuclease H at 37°C for 2 hours in their respective buffers. Before analysis by western blotting the samples were incubated with SDS loading buffer with 100 mM DTT. ABCG2 was visualized using the monoclonal antibody BXP-21 (1:1000), and IL2Rα was detected with mouse-IL2Rα antibody (1:200).

Results
To obtain a loss-of-function mutant of ABCG2 we mutated the conserved Walker A lysine to methionine (K86M). The ABCG2-K86M mutant was tagged at the N-terminus with the hemagglutinin (HA) epitope whereas ABCG2-wt was tagged with a MYC epitope. All constructs including untagged ABCG2-wt were expressed in HEK293 cells and the resulting cell lines were analyzed by SDS-PAGE and western blotting of membranes using a polyclonal antibody Ab-405 (Litman et al., 2002) directed against an intracellular epitope of ABCG2. As shown in Fig. 1A, this revealed clear expression of all constructs. The epitope tags did not alter apparent expression; however, the expression levels of the K86M mutants were somewhat lower than those observed for the wild-type constructs (Fig. 1A).

The activity of ABCG2-K86M in comparison to ABCG2-wt was first assessed by measurement of ATPase activity. Whereas the ABCG2-wt membrane fraction showed dose-dependent and saturatable stimulation of ATPase activity in response to increasing concentrations of the substrate prazosin (EC50=3.5 µM; Vmax=7.6 nmol/minute/mg protein), no stimulation of the ATPase activity was detected in membranes from cells expressing ABCG2-K86M and the basal ATPase activity was comparable to that of the empty HEK293 cells (Fig. 1B). We also assessed activity of the different constructs by measuring the sensitivity of the stably transfected cell lines to the chemotherapeutic agent mitoxantrone. Cells expressing ABCG2-wt or ABCG2-wt-MYC showed increased resistance to mitoxantrone as reflected in a sevenfold increase in IC50 value as compared to that observed for non-transfected cells (0.36 µM and 0.29 µM, for ABCG2-wt or ABCG2-wt-MYC expressing cells compared to 0.05 µM in non-transfected cells) (Fig. 1C). In contrast, ABCG2-K86M and ABCG2-K86M-HA displayed sensitivity comparable to that of non-transfected cells consistent with loss of function with IC50 values for mitoxantrone of 0.047 µM and 0.043 µM, respectively (Fig. 1C). Finally, we assessed transport activity directly by flow cytometry using BODIPY-prazosin as substrate. These results further confirmed that ABCG2-K86M was non-functional. Cells expressing ABCG2-wt and ABCG2-wt-MYC efficiently expelled the substrate; however, this was not the case for cells expressing ABCG2-K86M or ABCG2-K86M-HA, which displayed transport activity comparable to non-transfected cells (Fig. 2). Note that we observed no functional consequences of tagging the transporter in either the mitoxantrone resistance assay or in the flow cytometry assay (Fig. 1C, Fig. 2).

It has been suggested that the half-transporter ABCG2 exists as a homodimer (Kage et al., 2002; Litman et al., 2002). We wanted to further test this hypothesis and also analyze whether the K86M mutation altered the oligomerization properties of ABCG2. Accordingly, we coexpressed ABCG2-wt-MYC with ABCG2-K86M-HA as well as we coexpressed ABCG2-wt-MYC with ABCG2-wt tagged with HA instead of MYC. ABCG2-wt-HA displayed similar functional properties as ABCG2-wt and ABCG2-wt-MYC (data not shown). To test interaction between the coexpressed constructs we took advantage of the added epitope tags and performed co-immunoprecipitation experiments. Both ABCG2-wt-HA and ABCG2-K86M-HA co-immunoprecipitated with ABCG2-wt-MYC (Fig. 3, lanes 1,2). This suggests that ABCG2 exists in
an oligomeric complex and that mutation of Lys86 does not affect the oligomerization properties of the transporter. In the experiment shown in Fig. 3, the wt-HA band appears less intense than would be expected. However, we did not see the difference between the cell lines as a general phenomenon in our immunoprecipitations and in all our other experiments equal amounts of wt-HA and K86M-HA were precipitated (data not shown). The same cell lysates were also visualized with the HA antibody (Fig. 4). This blot further supports the fact that wild-type and mutant HA are expressed in equal amounts in the two different cell lines.

Several controls were included to exclude non-specific interactions in the co-immunoprecipitation assay; cells transfected with either ABCG2-wt-MYC or ABCG2-K86M-HA showed no crossreactivity between the two tags (Fig. 3, lanes 3,4). We also performed immunoprecipitation by mixing lysates from cells expressing the individual constructs; however, we observed no evidence for an interaction under these conditions indicating that oligomerization only occurs if the transporters are expressed within the same cell (data not shown).

In the absence of the reducing agent DTT, ABCG2 migrates on SDS-PAGE corresponding to the size of a dimer with a molecular mass of approximately 150 kDa (Fig. 4). This suggests that ABCG2 exists as a disulfide-linked dimer. To exclude the possibility that any disulfide bridges were formed during the extraction procedure we added the sulfhydryl-alkylating reagent N-ethylmaleimide at a concentration of 10

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Fig. 1. The K86M mutation in ABCG2 results in a non-functional transporter. (A) Protein expression analyzed on isolated membrane fractions from control (empty) HEK293 cells, or HEK293 cells stably expressing ABCG2-wt, ABCG2-wt-MYC (ABCG2-wt-cmyc), ABCG2-K86M and ABCG2-K86M-HA. The membrane fractions were analyzed by western blotting using the polyclonal antibody Ab-405 (Litman et al., 2002). (B) ATPase activity assay on isolated membrane fractions. The ATPase activity was measured as release of inorganic phosphate using a colorimetric assay. Vanadate-sensitive drug stimulated ATPase activity (mean±s.e.m., n=6) was measured with increasing concentrations of prazosin (0-50 µM) on ABCG2-wt, ABCG2-K86M and empty HEK293 cells. The experiment shown is representative of at least three other independent experiments. (C) Cytotoxicity assay was used to detect resistance of the stably transfected HEK293 cells to mitoxantrone. Stably transfected cells were incubated for 3 days with mitoxantrone and fixed in 50% TCA. Sulforhodamine B staining was used to detect survival of ABCG2-wt, ABCG2-wt-MYC (ABCG2-wt-cmyc), ABCG2-K86M, ABCG2-K86M-HA and empty HEK293 cells. The experiment shown (mean±s.e.m., n=3) is representative of four independent experiments.

Fig. 2. HEK293 cells transfected with ABCG2-K86M display no transport activity. The cells were incubated for 30 minutes at 37°C (accumulation) with 100 nM BODIPY-prazosin in the presence or absence of the specific ABCG2 inhibitor Fumitremorgin C (FTC) (5 µM). The cells were washed and incubated with or without 5 µM FTC for 60 minutes at 37°C (efflux). The histograms show intracellular fluorescence after accumulation and efflux of BODIPY-prazosin (100 nM) in the presence (broken line) or absence (unbroken line) of 5 µM FTC. Fluorescence was measured (FL-1) after excitation with a 488 nm argon laser.
mM to the lysis buffer as well as to the SDS-gel loading buffer to inhibit spontaneous formation of disulfide-bridges irrelevant for the native structure of the protein. Under these conditions we still observed disulfide-bridge linked dimerization of ABCG2 in all tested cells (Fig. 4). Altogether, this suggests that both ABCG2-wt and ABCG2-K86M form disulfide-bridge linked dimers. Note, that in the samples treated with DTT we observed an apparent decrease in signal on the western blot when using MYC antibody but not when using the HA-antibody. We have no explanation for this phenomenon that is also observed when we use a monoclonal anti-ABCG2 antibody. It should be mentioned that the same observation has been made previously (Kage et al., 2002).

Next, to investigate whether ABCG2 is a functional homodimer we needed to assess whether both half-transporters have to be functional in order to form a functional transporter. We examined this by ATPase activity measurements on isolated membrane fractions from cells coexpressing ABCG2-wt-MYC and ABCG2-K86M-HA, cells coexpressing ABCG2-wt-MYC and ABCG2-wt-HA, or cells expressing ABCG2-wt alone. Using a monoclonal anti-ABCG2 antibody directed against an intracellular epitope of ABCG2 we found an increase in expression of total ABCG2 in the co-transfected cells (Fig. 5A). We also found based on densitometry analysis of several western blots that the co-transfected cells expressed equal amounts of HA-tagged transporter (ABCG2-K86M-HA or ABCG2-wt-HA) and MYC-tagged transporter (ABCG2-wt-MYC) (Fig. 5B).

Upon stimulation with increasing concentrations of prazosin (Fig. 5C), cells expressing ABCG2-wt alone showed activation of ATP hydrolysis ($EC_{50}=4.4\pm0.8 \mu M$; $V_{\text{max}}=8.2\pm0.2$ nmol/minute/mg protein). In cells coexpressing ABCG2-wt-MYC and ABCG2-wt-HA the $V_{\text{max}}$ was larger than in cells expressing ABCG2-wt alone although $EC_{50}$ remained constant ($EC_{50}=3.1\pm0.3 \mu M$; $V_{\text{max}}=10.2\pm0.1$ nmol/minute/mg protein). However, in cells coexpressing ABCG2-wt-MYC and ABCG2-K86M-HA we found a markedly lower $V_{\text{max}}$ both compared to cells expressing ABCG2-wt alone and to cells coexpressing ABCG2-wt-MYC and ABCG2-wt-HA ($EC_{50}=3.8\pm0.9 \mu M$; $V_{\text{max}}=5.2\pm0.1$ nmol/minute/mg protein; $P<0.0001$, unpaired $t$-test, $V_{\text{max}}$ compared to either ABCG2-wt alone or ABCG2-wt-MYC/ABCG2-wt-HA). The decrease in activity upon co-transfection of ABCG2-wt and ABCG2-K86M is consistent with a dominant-negative effect caused by the formation of an inactive wt/K86M complex and, accordingly, supports the fact that ABCG2 is a functional dimer. We should note, however, that based on the dominant-negative effect observed, we cannot exclude the fact that the transporters may exist and function as a higher order form than a dimer.

We also investigated the subcellular localization of ABCG2-wt in comparison to ABCG2-K86M. Interestingly, immunocytochemical studies showed a distinct staining pattern of ABCG2-wt in comparison to ABCG2-K86M. ABCG2-wt staining was localized almost entirely to the plasma membrane of the cells (Fig. 6A-C). In contrast, ABCG2-K86M staining was punctate, and found almost exclusively in an intracellular compartment (Fig. 6D,E); hence, mutation of Lys86 might not only affect the functional properties of the transporter but also its cellular targeting. In order to obtain a quantitative measurement for the decrease in surface expression of the K86M mutation compared to ABCG2-wt, the cell lines were

![Fig. 3. ABCG2-wt and ABCG2-K86M co-immunoprecipitate. Total cell lysates from stably transfected HEK293 cells were immunoprecipitated (IP) with anti-MYC antibody and protein G agarose. All cell lysates contained 5 mM N-Ethylmaleimide to inhibit spontaneous cysteine oxidation. The eluate was analyzed by immunoblotting (IB) with either anti-HA or anti-MYC (cmyc) antibodies. Cell lysates were loaded on SDS-PAGE as positive controls for expression of both constructs (lane 7, 8). Singly transfected cells were used as negative controls (lane 3, 4).](image-url)

![Fig. 4. ABCG2 exists as a disulfide-linked dimer. Total cell lysates from stably transfected HEK293 cells were analyzed by SDS-PAGE and western blotting. To avoid formation of non-natural disulfide bridges 10 mM N-ethylmaleimide was included in both the lysis buffer and in the SDS loading buffer. The samples were incubated in the SDS loading buffer with or without 100 mM DTT as indicated for 30 minutes before application to the gel. For the immunoblotting we used both HA antibody (upper panel, IB:HA) and MYC antibody (lower panel, IB:cmyc).](image-url)
analyzed by flow cytometry. In these experiments, the cells were stained with a phycoerythrin (PE)-conjugated ABCG2-antibody directed against an extracellular epitope of ABCG2. The cells were also stained with propidium iodide to exclude dead cells with a damaged plasma membrane. The experiments showed that the apparent surface expression of ABCG2-K86M was ~30% of that observed for the wild type consistent with our immunofluorescence data (Fig. 7A). In cells coexpressing ABCG2-wt-MYC and ABCG2-wt-HA or ABCG2-wt-MYC and ABCG2-K86M-HA we observed that the apparent surface expression in both cases was 30-40% higher than in cells only expressing ABCG2-wt-MYC. A possible explanation for the similar expression in both cell lines could be that the wild type is capable of rescuing the impaired surface targeting of the mutant via an oligomeric interaction. This was supported by immunostaining cells expressing K86M alone in comparison to cells expressing both K86M-HA and wt-MYC. As shown in Fig. 7B the vast majority of anti-HA staining was found intracellularly in cells expressing only K86M-HA whereas in the cells expressing K86M-HA together with wt-MYC we observed increased plasma membrane anti-HA staining.

To determine the subcellular compartment in which ABCG2-K86M was localized we performed a series of co-stainings using the anti-ABCG2 antibody together with markers for different cellular compartments. We found similar staining patterns for ABCG2-K86M and anti-calnexin, a marker for the endoplasmic reticulum (ER) (Fig. 8B). In contrast, we found no evidence for colocalization with giantin (a cis-Golgi marker, Fig. 8D) and Rab5 (an endosome marker, Fig. 8F). Altogether, we conclude that ABCG2-K86M is mainly localized to the ER although a small percentage is localized to the plasma membrane.

To verify our localization results we analyzed the glycosylation state of ABCG2-wt and ABCG2-K86M. We treated total cell lysates from the two cell lines with either PNGase F (N-glycosidase F) or Endo H (endonuclease H). PNGase F removes all N-linked glycosylation and treatment with PNGase F caused a considerable and similar reduction in size of the proteins demonstrating that both the wild type and mutant are glycosylated to the same degree (Fig. 9A, lanes 5 and 7). The glycosylation was, however, insensitive to Endo H, which cleaves off only the high mannose type of asparagine-linked oligosaccharides added in the ER but not complex forms of N-glycosylation added later in the processing pathway (Fig. 9A, lanes 1 and 3). As a positive control for the effect of Endo H we used IL-2Rα (Richardson et al., 1995). The immature
and ER-localized form of this protein was sensitive to treatment with Endo H (Fig. 9B). These data imply that ABCG2-K86M is processed beyond the ER and possibly to cis-Golgi from where it is retrieved again to the ER where it is mainly detected.

**Discussion**

In this paper we present evidence that ABCG2 is a structural as well as a functional oligomer. Furthermore, we show that mutation to methionine of the highly conserved lysine in Walker A (K86M) not only results in a non-functional transporter but also has a profound effect on targeting of the transporter to the cell surface. In contrast, we observed no effect on oligomerization of the transporter. This supports the notion that formation of an oligomeric complex is not dependent on functional NBD domains although intact NBDs appear to be required for efficient surface targeting of the transporter.

Mutation of the highly conserved Lysine in Walker A has been studied in several ABC transporters (Davidson and Sharma, 1997; Lapinski et al., 2001; Ozvegy et al., 2002; van Veen et al., 2000). The mutation renders the transporter inactive with respect to ATP hydrolysis (Muller et al., 1996; Szabo et al., 1998) and can impair ATP binding (Lapinski et al., 2001). In ABCG2, it has been demonstrated by expression in Sf-9 insect cells that K86M is functionally inactive but still capable of binding ATP (Ozvegy et al., 2002). Accordingly, we chose K86M to address whether ABCG2 is a functional oligomer and established co-transfected stable HEK293 cell lines expressing the wild type alone, K86M alone or wild type and K86M half-transporter together. Analysis of the co-transfected cells with respect to ATPase activity suggested a dominant-negative activity of the K86M mutant on wild-type activity, i.e. membranes from cells coexpressing wt-MYC and K86M-HA displayed less ATPase activity as compared to cells expressing the wild type alone and only about 40% of the ATPase activity was observed in cells expressing both wt-HA and wt-MYC (Fig. 5). In the ideal situation the dominant-negative effect should be a 75% reduction in activity and not
the ~60% we observed (Fig. 5). One possible explanation for this apparent discrepancy is that we have used stably transfected pool clones and accordingly cannot assume that the wild type and K86M are expressed in exactly equal amount in all cells. It is also possible that because the wild type and K86M preferentially are targeted to the ER and cell surface, respectively, we might not obtain the ideal distribution of 50% wt/K86M dimers, 25% wt/wt dimers and 25% K86M/ K86M.

The presence of a dominant-negative effect supports the idea that the inactive K86M mutant is capable of oligomerizing with the wild type resulting in a non-functional wt/K86M transporter protein complex and, thus, that at least two functional NBDs are necessary for proper ATP hydrolysis. Interestingly, a dominant-negative effect on drug sensitivity has been reported for ABCG2 (Kage et al., 2002) upon mutation of Leu554 situated in transmembrane segment 5 to a proline (L554P). It was, however, not assessed in this study whether the dominant-negative effect of this very different mutation was the result of disrupted oligomerization, interferences with substrate binding or impaired targeting of the mutant (Kage et al., 2002).

Previously, dominant-negative effects have also been obtained in the bacterial multidrug resistance ABC half-transporter LmrA upon mutation of the lysine corresponding to Lys86 in ABCG2. A dominant-negative effect was observed when the mutant and wild-type half-transporters were linked together as a fusion protein (van Veen et al., 2000). In the ‘full’ transporter Pgp it has similarly been observed that mutation of the lysine in one of the two NBDs results in an inactive

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**Fig. 8.** The ABCG2-K86M mutant resides in the ER. Immunocytochemistry co-stainings were performed using an Anti-ABCG2 antibody and antibodies directed against different cellular organelles. Left-hand column, ABCG2 staining; middle column, organelle staining; and right-hand column, overlay of the single stainings. (A,B) Anti-Calnexin (ER), (C,D) Giantin (cis-Golgi) and (E,F) Rab5 (early endosomes). Secondary antibodies were Alexa 488 and Alexa 568. The pictures were obtained by confocal laser-scanning microscopy on a Zeiss LSM 510 confocal microscope.

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**Fig. 9.** ABCG2-K86M is glycosylated to the same degree as ABCG2-wt. (A) Deglycosylation of ABCG2 protein with PNGase F or endonuclease H (Endo H). Total cell lysates of ABCG2-wt or ABCG2-K86M were treated with denaturation buffer for 10 minutes at 100°C. 15 µg total protein were incubated for 2 hours at 37°C with 100 mU enzyme. Samples were analyzed by SDS-PAGE and western blotting using the monoclonal anti-ABCG2 antibody BXP-21. (B) Positive control for endonuclease H activity. Immature IL2Rα (at 35-40 kDa) is sensitive to endonuclease H and is fully deglycosylated whereas the mature IL2Rα (50-60 kDa) is insensitive to endonuclease H treatment.
transporter protein (Muller et al., 1996). Overall, our current data provide further support for a general mechanism of action for multidrug resistance ABC transporters requiring two active NBDs. Putatively, this might involve a two-cylinder engine mechanism as proposed for LmrA (van Veen et al., 2000).

We also assessed the oligomerization properties of ABCG2-wt and ABCG2-K86M by co-immunoprecipitation. This provided evidence for cellular interaction both between two differently tagged wild-type transporters (wt-HA and wt-MYC) and between wt-MYC and K83M-HA. Moreover, we obtained evidence that oligomerization of ABCG2-2 is assisted by intermolecular disulfide bridges (Fig. 4). Interestingly, ABCG2 contains three extracellular cysteines according to its predicted topology (Cys592, Cys603 and Cys608). It can therefore be speculated that one or more of these cysteines are involved in the formation of intermolecular disulfide bridges between two individual half-transporter molecules. How the cysteines interact and how this influences transporter function remains to be clarified. One recent observation showed, however, that β-mercaptoethanol reduces the ABCG2 dimer without having any effect on the observed methotrexate transport (Mitomo et al., 2003). We should also note that although it was recently reported that ABCG2 mainly exists as a homotetramer (Xu et al., 2004), we found no evidence for such oligomers in our experimental set-up. One possibility is that the transporter exists as a disulfide-linked dimer and that this dimer can dimerize resulting in a tetrameric structure. The interaction between the dimers is probably not resistant to SDS and, thus, the tetrameric form will dissociate into dimers during denaturing SDS gel electrophoresis.

As a part of our characterization of the different ABCG2 expressing cell lines we performed immunocytochemistry. Surprisingly, we observed a striking difference between the localization of ABCG2-wt (plasma membrane) and ABCG2-K86M (ER). To the best of our knowledge this is the first report showing that a functional NBD is required for proper cellular sorting of an ABC half-transporter. A previous study on ABCG2-K86M suggested normal surface expression in S9 insect cells (Ozvegy et al., 2002). However, the processing machinery of insect cells probably differs substantially from that in mammalian cells providing a possible explanation for this apparent discrepancy.

Immunocytochemical staining suggests that the K86M mutant resides in the ER, either because of retention or retrieval of the transporter to this compartment. In this context it is interesting to note that western blot analysis comparing the wild type and K86M showed that they both migrate identically in the gel. Furthermore, treatment with PNGase F caused a similar reduction in size demonstrating that both wild type and mutant were glycosylated to the same degree. The glycosylation was, however, insensitive to Endo H indicating that both ABCG2-wt and ABCG2-K86M has been processed beyond the ER. This supports a scenario in which the K86M mutant is processed to the Golgi or the intermediary compartment and subsequently retrieved to the ER as part of a putative quality control mechanism.

The available structural data on ABC transporters gives rise to speculation regarding how the K86M mutation affects surface targeting. The NBDs have been crystallized for several transporters both alone and in full transporters (Hopfner et al., 2000; Hung et al., 1998; Locher et al., 2002). The growing consensus is that the NBDs share an interface with walker A of one NBD opposite the C signature of the other NBD with the ATP binding site buried in the dimer interface (Chang, 2003; Chen et al., 2003a; Shilling et al., 2003). As it was previously shown that the ABCG2-K86M mutation can still bind but not hydrolyze ATP (Ozvegy et al., 2002), a conceivable scenario might be that hydrolysis of ATP facilitates a closed structure of the NBDs; hence, a transporter unable to hydrolyse ATP is likely to have a markedly looser structure. Since the K86M mutation directly affects the ATP binding site, the NBD dimerization interface could be affected leading to impaired surface targeting followed by retrieval to ER. We still observe dimerization of the transporter when mutating K86M, suggesting that dimer formation is likely to be dependent on the transmembrane domains as well as on disulfide bridges formed between cysteines present in the predicted extracellular loops. Indeed, these dimers might already be formed in the ER and conceivably their formation is necessary for ER export as it has been shown for the G family members G5 and G8 (Graf et al., 2002; Graf et al., 2003).

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