Recombinant Synthesis of Human ABCG2 Expressed in the Yeast *Saccharomyces cerevisiae*: an Experimental Methodological Study

Anna Jacobs · Dana Emmert · Svenja Wieschrath · Christine A. Hrycyna · Michael Wiese

Abstract Human ABCG2 is an efflux protein belonging to the ATP-binding cassette transporter superfamily. It is expressed in the plasma membrane of different cell types performing various physiological functions. It is the most recently discovered MDR transporter and its structure and function are still not well understood. Thus, expression and functional reconstitution of the protein in different variants and from different sources are important steps for its further investigation. In this work we describe a recombinant synthesis of human ABCG2 R482G from *S. cerevisiae*. We expressed the human ABCG2 R482G variant in *S. cerevisiae* and purified the protein from total yeast membranes. Using a panel of sixteen detergents, we analyzed the efficiency of extraction of ABCG2 from membranes by SDS–PAGE and immunoblot analysis. Based on these results, three detergents were selected for further purification studies and two of them, n-octyl-β-D-glucopyranoside and n-dodecyl-β-D-maltopyranoside, yielded functional protein after reconstitution into liposomes. We show here the first example of purified and reconstituted ABCG2 expressed in *S. cerevisiae* retaining drug-stimulated ATPase activity.

Keywords ABCG2 · Breast cancer resistance protein · Expression · Yeast · Purification

Abbreviations

MDR	Multidrug resistance
ABC	ATP-binding cassette
BCRP	Breast cancer resistance protein
ABCP	Placental ABC protein
MXR	Mitoxantrone resistance protein
MRP	Multidrug resistance associated protein
MM	Minimal media
PMSF	Phenylmethansulfonyl fluoride
DFP	Diisopropyl fluorophosphate
PVDF	Polyvinylidene fluoride
HRP	Horseradish peroxidase
Ni-NTA	Ni^{2+}-nitrilotriacetate
CHAPSO	3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate
DM	n-decyl-β-D-maltopyranoside
DDM	n-dodecyl-β-D-maltopyranoside
OG	n-octyl-β-D-glucopyranoside
FC-12	Fos-choline 12
FC-13	Fos-choline 13
FC-14	Fos-choline 14
FC-15	Fos-choline 15
FC-16	Fos-choline 16
PMA1	Plasma membrane H^+-ATPase
PDR	Pleiotropic drug resistance
PGK	Phosphoglycerate kinase

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1 Introduction

Multidrug resistance (MDR) occurring in human breast cancer cells and several other solid tumour cell lines, which do not show expression of ABCB1 (P-glycoprotein) or transporters from the ABCC family (MRPs), has been implicated strongly with ABCG2 [8, 18, 25, 43]. ABCG2,

also known as breast cancer resistance protein (BCRP)/placental ABC protein (ABCP)/mitoxantrone resistance protein (MXR), is the latest MDR transporter to be discovered [1, 8, 30].

A wide variety of cytostatic drugs are extruded out of tumour cells by ABCG2, thus lowering their intracellular levels below the effective concentration [7, 11, 40]. ABCG2 is also expressed in normal tissues at pharmacologically relevant barriers throughout the human body, such as the apical membrane of placental syncytiotrophoblasts, the bile canalicular membrane of hepatocytes, the luminal membranes of epithelial cells in the small intestine and colon or in ducts and lobules of the breast [23, 25]. Its distribution and functional characteristics point to an essential role of ABCG2 in protecting the human body from potentially toxic substances by limiting uptake and absorption as well as mediating hepatobilary elimination [28, 39, 48].

Overexpression of ABCG2 was also found in so-called human cancer stem cells, which may exist as a small side-population in tumours [4, 50]. Cancer stem cells are thought to escape chemotherapy treatment, thus being responsible for disease reoccurrence. It has also been proposed that the down- and up-regulation of ABCG2 in cancer stem cells contributes to the proliferation of the tumour [4].

ABCG2 is comprised of 655 amino acids (fully glycosylated: $\sim$ 72 kDa) forming an N-terminal ATP-binding domain and a C-terminal transmembrane region containing 6 transmembrane segments. A homodimer connected via disulfide bonds has been proposed to be the functional form [3, 16, 21, 24], however higher organized oligomers have also been observed [29, 51]. Mutation of arginine-482 to threonine or glycine considerably extends the spectrum of transported substrates. The variants R482T or R482G transport additional substrates such as rhodamine 123 and doxorubicin, whereas the recognition of other substrates such as Hoechst 33342 and pheophorbide A remains unaffected [10, 17]. One of these variants (R482G) was chosen for purification experiments described in this manuscript showing the above mentioned broader substrate recognition. Using this mutant would be beneficial for the investigation of protein function and future binding studies as it allows the involvement of experimental functional assays employing the fluorescent dye rhodamine 123.

Sufficient expression of ABCG2 in the starting material is vital for protein purification. Endogenous expression levels in human tissues are low, therefore only over-expressing cultured cell lines or high-yield heterologous expression systems seem to be suitable for this approach. Although many cultured drug-resistant cancer cell lines have been established, the use of these systems for purification is limited due to expense or the inability to mutate the protein of interest. In the past years several heterologous expression systems for ABCG2 have been developed, including *Lactococcus lactis* [5, 19], transiently transfected HEK 293 cells [45] and oocytes from *Xenopus laevis* [31]. However, purification from the above mentioned systems has not been demonstrated to date. Membrane preparations obtained from *Spodoptera frugiperda* (Sf9) and *Trichoplusia ni* (HighFive^{TM}) insect cells infected with a human ABCG2-expressing baculovirus contain large amounts of ABCG2 and appear to be useful for protein purification [29, 36, 37]. But major drawbacks of this system are the expensive cell culture techniques as well as the requirement to produce a new virus for each mutant.

In comparison to cultured cell lines, yeast expression systems involve simple and cost-effective media and culture techniques, and large amounts of membranes can be easily produced as starting material for purification. Another advantage of using yeast for expression of the membrane protein ABCG2 is the presence of an eukaryotic membrane biogenesis and trafficking machinery [12]. The yeast *Pichia pastoris* has already been used to express ABCG2, but only 10% of the clones expressed the transporter and the expression level was very heterogenous [26]. *Saccharomyces cerevisiae* (*S. cerevisiae*) strains have been used previously to successfully express and purify large quantities of functional P-glycoprotein (P-gp) [14, 27].

An expression system for P-gp in the ergosterol-deficient yeast strain LPY11 [34] has already been established [46]. We have tried to apply the same system to human ABCG2 (R482G variant). In this study we present the first report of successful purification and functional reconstitution of human ABCG2 from *S. cerevisiae* membranes. The expressed protein was solubilized with n-octyl-β-D-glucopyranoside (OG), fos-choline 16 (FC-16) and n-dodecyl-β-D-maltopyranoside (DDM) and reconstituted into proteoliposomes. We were able to show function by stimulation of ATPase activity with prazosin and other known substrates of ABCG2 (R482G) such as sulfasalazine and progesterone [15, 19].

In summary a heterologous expression system for the human ABC transporter ABCG2 was established in the ergosterol-deficient *S. cerevisiae* strain LPY11. Major advantages over other expression systems such as Sf9 insect cells are the cost-effective media and simple culture techniques of yeast, therefore large quantities of yeast membranes as starting material for purification experiments can be obtained without much effort. This system could also be readily applied for studies of different ABCG2 mutants, and large amounts of purified ABCG2 would be a useful tool for future mechanistic, biophysical and structural studies of this ABC transporter.

2 Materials and Methods

2.1 Expression Vector pCHH10m3N-ABCG2

Human ABCG2 cDNA (R482G variant) was cloned into the pCHH10m3N plasmid [2], where the expression was under control of the constitutive phosphoglycerate kinase (PGK) promoter. Two tags were fused to the N-terminus of ABCG2: a his-tag composed of ten histidine residues followed by a myc-tag consisting of three myc epitopes.

2.2 Strain and Media

The *S. cerevisiae* ergosterol-deficient strain LPY11 (ATCC 201842, MATa erg6::LEU2 leu2 his3 ura3-52), generously provided by Dr. Al-Shawi (University of Virginia), was transformed with the plasmid pCHH10m3 N-ABCG2. Transformed cells (LPY11-ABCG2) were grown in minimal media (MM) containing 0.67% yeast nitrogen base without amino acids, 2% (w/v) D-glucose and an amino acid mixture lacking L-leucine and uracil. Untransformed yeast cultures (LPY11) were grown in media supplemented with 0.18 mM uracil. Solid media additionally contained 2% agar.

2.3 Yeast Culture and Transformation

LPY11 cells were transformed by lithium acetate using the Yeastmaker yeast transformation system (CLONTECH Laboratories) and LPY11-ABCG2 clones were obtained by auxotrophic selection. Cells were pre-cultured in 50 ml MM at 30 °C with constant agitation (200 rpm) until 1 OD_{600}. This pre-culture was then diluted to 0.1 OD_{600} in 500 ml MM and again cultured until 1 OD_{600}. After diluting the cells again to 0.1 OD_{600} they were cultured in up to 10 L MM in a fed batch procedure to 4 OD_{600}.

2.4 Isolation of Membranes

For isolation of the plasma membrane, yeast cultures were harvested by centrifugation at 10,000 g for 10 min (4 °C) and processed as described in [14] with the following modifications: Instead of a French pressure cell a bead-beater was used for mechanical disruption of the yeast cells. Furthermore the protease inhibitor cocktail containing benzamidine, leupeptin, pepstatin A, PMSF and DFP was used in all buffers throughout the membrane isolation process, not only in the homogenization buffer.

All procedures were performed at 4 °C. Cells were resuspended in homogenization buffer (50 mM Tris–HCl, pH 7.5, 0.3 M sucrose, 5 mM EDTA, 1 mM EGTA, 5 mg/ml bovine serum albumin (BSA), 1 mM β–mercaptoethanol, 1 mM benzamidine, 1 μM leupeptin, 2 μM pepstatin A, 1 mM PMSF, and 1 mM DFP). After mechanical cell disruption with a bead-beater (BioSpec Products) the lysate was centrifuged at 2,900 g for 5 min and thereafter at 14,000 g for 20 min. The resulting supernatant was ultracentrifuged at 200,000 g for 80 min, and pelleted membranes were resuspended in washing buffer (10 mM Tris–HCl, pH 7.5, 10% glycerol (v/v), 1 mM EGTA, 2 mM β–mercaptoethanol, 1 mM benzamidine, 1 μM leupeptin, 2 μM pepstatin A, 1 mM PMSF, and 1 mM DFP), homogenized with a glass homogenizer, and centrifuged again at 200,000 g for 80 min. For storage at −80 °C, membranes were resuspended in storage buffer (10 mM Tris–HCl, pH 7.5, 25% glycerol (v/v), 1 mM EGTA, 2 mM β–mercaptoethanol, and 1 mM PMSF).

2.5 Solubilization Screen for Recombinant Human ABCG2

Detergent solubilization of ABCG2 from yeast membranes was tested with 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO), brij 35, brij 58, triton-X-100, n-decyl-β-D-maltopyranoside (DM), n-dodecyl-β-D-maltopyranoside (DDM), n-octyl-β-D-glucopyranoside (OG), fos-choline 12 (FC-12), fos-choline 13 (FC-13), fos-choline 14 (FC-14), fos-choline 15 (FC-15), fos-choline 16 (FC-16), cymal 5, cymal 6, and cymal 7. Membranes were treated with solubilization buffer (modified from Figler et al. [14]) (25 mM Tris–HCl, pH 8, 20% glycerol (v/v), 50 mM Na_2SO_4, 1 mM ATP, 1 mM $MgSO_4$, 1 mM β–mercaptoethanol, 5 mM imidazole, pH 8, 1 mM PMSF, 2 μM pepstatin A, 1 μM leupeptin, and 1 mM benzamidine) for 60 min with a protein concentration of 5 mg/ml containing 1% (w/v) of detergent with gentle shaking. Soluble and insoluble fractions were separated by ultracentrifugation at 300,000 g for 30 min. The insoluble fraction was resuspended in detergent-free solubilization buffer. All steps were performed at 4 °C.

2.6 Mixed Lipid Stock

Mixed lipid stocks were prepared by solving 23% cholesterol (Sigma), 67% L-α-phosphatidylcholine from egg yolk (Avanti polar lipids) and 10% L-α-phosphatidic acid (sodium salt) from egg yolk (Avanti polar lipids) in chloroform. The solvent was evaporated in vacuum and dried lipids were resuspended in buffer (50 mM Tris–HCl, pH 7.4, 250 mM sucrose, 20% glycerol (v/v), 5 mM 6-aminohexanoic acid, 2% OG, and 1 mM NaN_3) to a final concentration of 50 mg/ml using 2.5 mm glass beads (BioSpec Products). Lipids were shaken gently over night and then frozen at −80 °C for storage. Mixed lipid stocks

were thawed and shaken gently for about 10 h at room temperature before use.

2.7 Purification and Reconstitution of Recombinant Human ABCG2

Purification of ABCG2 from crude yeast membranes was performed as described in the literature [14] with the following modifications: the initial protein concentration was increased, additionally the incubation times in the presence of detergent and of Ni–NTA were prolonged to optimize protein yield of the solubilization procedure.

All steps were performed at 4 °C. ABCG2 was solubilized in solubilization buffer containing 2% OG, 2% FC-16 or 1% DDM, respectively, at a protein concentration of 5 mg/ml with gentle shaking for 60 min. The suspension was centrifuged at 100,000 g for 70 min and the supernatant was supplemented to 200 mM Na_2SO_4. After adjustment of the pH to 8.0 using Tris base, solubilized proteins were incubated with Ni–NTA Superflow resin (Qiagen) for 5 h with gentle stirring (6.5 ml membranes per milliliter of resin). The resin was loaded onto a column (Bio-Rad) and washed with about 20 column volumes of washing buffer (25 mM Tris–HCl, pH 8.0, 20% glycerol, 200 mM Na_2SO_4, 2 mM ATP, 2 mM $MgSO_4$, 1 mM β–mercaptoethanol, 50 mM imidazole, pH 8.0, 1.4% (w/v) OG, 0.1% (w/v) mixed lipid stock, 1 mM PMSF, 2 μM pepstatin A, 1 μM leupeptin, and 1 mM benzamidine). To elute bound ABCG2 from the resin, 4 column volumes of elution buffer (25 mM Tris–HCl, pH 7.4, 20% glycerol, 50 mM Na_2SO_4, 2 mM ATP, 2 mM $MgSO_4$, 2 mM β–mercaptoethanol, 250 mM imidazole, pH 7.4, 1.4% (w/v) OG, 0.4% (w/v) mixed lipids, and 1 mM PMSF) were added and the eluate was collected. The purified protein was reconstituted into a defined lipid environment via dialysis against 70 volumes of dialysis buffer (50 mM Tris–HCl, pH 7.4, 1 mM EGTA, 5 mM 6-aminohexanoic acid, 1 mM DTT) for 16 h, with one buffer exchange after 11 h. Proteoliposomes were centrifuged at 186,000 g for 3 h, resuspended in dialysis buffer, frozen on dry ice in aliquots and stored at −80 °C. The lipid-to-protein ratio of the proteoliposomes was about 270:1 by weight.

2.8 Protein Quantification

The total amount of protein in the samples was determined by the amido black 10B protein assay described earlier by Kaplan et al. [22].

2.9 SDS PAGE, Staining and Western Blotting

Samples (2 or 5 μg protein per lane, or 15 μL for samples with very low protein content) were separated by a 7.5% SDS–polyacrylamide gel. The proteins were stained with alkaline silver or with sensitive colloidal Coomassie Brilliant Blue G-250 [9]. For immunostaining, proteins were transferred to PVDF membranes and ABCG2 was detected by staining with BXP-21 (1:1,000) or a myc antibody (1:200) followed by HRP-conjugated goat anti-mouse IgG (1:20,000) and visualized using Immobilon[TM] Western chemiluminescent HRP substrate (Millipore).

2.10 ATPase Activity Assay

The protocol for measuring the ATPase activity of purified ABCG2 is based on the colorimetric determination of inorganic phosphate (P_i) released by the hydrolysis of ATP [49]. The reaction volume of 250 μL contained up to 1.9 μg of purified protein and was incubated in assay buffer (20 mM HEPES, pH 7.0, 5 mM $MgSO_4$, 2 mM NaN_3, and 10 mM ATP) at 37 °C in the presence of various concentrations of test drugs or 1 mM sodium orthovanadate. Samples (50 μL) were taken at distinct time points (0, 20, 40, 60 min) and the reaction was stopped by the addition of ice-cold 8 mM EDTA (pH 8.0). Liberated P_i was detected using ammonium molybdate and malachite green solutions, and absorbance was measured at 610 nm. Linear regression of the four time points from each reaction was performed to calculate the slope s_r, which corresponds to the reaction rate. The specific activity of the protein was determined utilizing the slope of the calibration line (s_c) and the volume ($v_{protein}$) and concentration ($c_{protein}$) of the protein suspension using the following equation:

$$specific\ activity = \frac{s_r}{s_c} * \frac{5}{v_{protein} * c_{protein}}$$

Results are presented as relative vanadate sensitive ATPase activities (relative units). Concentration–response curves were generated by nonlinear regression applying a four-parameter logistic equation with variable slope (GraphPad Prism® 5.0 software). All experiments were performed at least two times in triplicate using the same lot of purified ABCG2 to avoid possible effects of lot to lot variation.

2.11 Mass Spectrometry

Samples were subjected to SDS–PAGE, followed by staining with Coomassie Brilliant Blue G-250 and tryptic in-gel digestion. The digestion procedure was performed as described in the literature [44] with several modifications. Briefly, protein bands of interest were excised and exposed to modified trypsin (Sigma) at a trypsin:protein ratio of approximately 2:1 (w/w) in 25 mM NH_4HCO_3 pH 8.0 at 37 °C overnight. Generated peptides were eluted successively with acetonitrile/formic acid/water 50:5:45 (v/v) and

100% acetonitrile. Eluted peptides were purified and desalted over a C18 reversed-phase mini column (Eppendorf), before mass spectrometric analysis. Electrospray ionization-mass spectrometry measurements were performed using an ESI-Q-qTOF (QSTAR XL, Applied Biosystems, Darmstadt, Germany) equipped with a nanospray ion source. Peptides were identified by MS Fit employing an in-silico tryptic digest of ABCG2 using the GPMAW 8.0 software (Lighthouse data).

2.12 Other Materials

All detergents were obtained from Anatrace, except for Brij 35 (Acros), Brij 58 (Acros) and Triton-X-100 (Sigma). The mouse monoclonal antibody BXP-21 was from Abcam, the HRP-conjugated goat anti-mouse IgG was from Calbiochem. All other chemicals, unless otherwise stated, were from Sigma or Applichem.

3 Results and Discussion

3.1 Expression of ABCG2 (R482G) in *S. cerevisiae*

The ABCG2 variant R482G was chosen, as it displays altered substrate specificity in comparison to wild-type (wt) ABCG2. In the literature this variant is characterized as a gain-of-function mutation for ATPase activity and for substrate transport [10, 17]. ABCG2 was successfully expressed using the pCHH10m3 N plasmid, where the expression is driven by the strong PGK promoter. The tag consisting of ten histidine residues followed by three myc epitopes was fused to the N-terminus of the human ABCG2 cDNA. In the literature it was described that a C-terminal tag was detrimental to function and expression [29]. The expression vector described above was transformed into the ergosterol-deficient yeast strain LPY11. The ERG6 gene, which encodes a sterol methyltransferase converting zymosterol to fecosterol has been deleted from this strain [34]. This strain has already been successfully used in our laboratory as an expression system for P-glycoprotein [46].

Individual LPY11-ABCG2 clones were cultured and the expression of ABCG2 in isolated membranes was confirmed using SDS–PAGE and immunoblotting. All clones expressed ABCG2 and showed similar expression levels (data not shown), which renders the *S. cerevisiae* strain LPY11 a useful expression system for this ABC transporter. An immunoblot comparing wt membranes from the control strain (LPY11-wt) with membranes from transformed cells (Fig. 1) demonstrated the expression of ABCG2 in the LPY11-ABCG2 strain. The protein was produced as two species with slightly different migration behaviour on SDS–PAGE. This result is consistent with
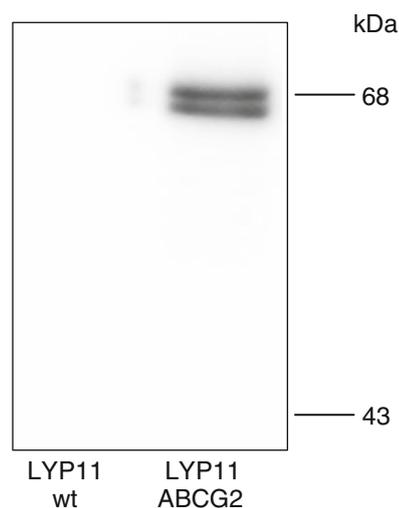
Fig. 1 Expression of ABCG2 in LPY11-wt and -ABCG2 yeast membranes revealing the expression of two protein species. It was suggested that the upper species is the mature functional protein while the lower species is immature and inactive [36]. Membranes were separated by a 7.5% SDS–PAGE and ABCG2 expression was analyzed by immunoblot analysis of LPY11-wt and -ABCG2 yeast membranes (5 μg per lane) using the monoclonal antibody BXP-21

previous observations that ABCG2 expressed in Sf9 cells also shows two bands by immunoblotting. Pozza and co-workers suggested that the upper species is the mature functional protein while the lower species is immature and inactive [36]. The apparent molecular weight of the upper species was ∼68 kDa and matched the predicted weight from the ABCG2 protein sequence, indicating that the protein is probably under-glycosylated when expressed in *S. cerevisiae*. It has been previously shown that ABCG2 has a glycosylation site at asparagine 596, but that the glycosylation-deficient protein is still normally expressed and active [6, 26, 33].

To further characterize the new strain, its growth rate was determined in comparison to the wt strain in liquid medium. The doubling time of cells expressing ABCG2 (2.2 h) was longer than that of wt cells (1.4 h). These results suggest that ABCG2 expression negatively affects yeast growth. Similar results have been published for P-glycoprotein [14, 27], suggesting that this may be a general consequence of the forced expression of foreign proteins in yeast.

We could not ascertain the function of the expressed protein at this point, because assessing the functionality of expressed ABCG2 in living yeast cells is difficult. Its activity can be masked by a variety of other multidrug transporters from the pleiotropic drug resistance (PDR) network [20, 41]. The best characterized of these plasma membrane proteins are the related ABC transporters Pdr5p and Snq2p. These proteins are presumed among others to mediate the translocation of anticancer drugs [20].

Unfortunately, to date, little is known about the specificity of yeast transporters for chemotherapeutic agents that are also substrates of ABCG2 [12], therefore these transport proteins may be able to mask the specific activity of ABCG2 in living yeast.

Crude yeast membranes were also not suitable for functional studies by ATPase assays, as they additionally contained other yeast specific endogenous ATPases, for example the plasma membrane H^+-ATPase (PMA1) [13], thus producing high background in these assays. Consequently it was necessary to solubilize and purify ABCG2 to determine the specific ATPase activity of the expressed protein.

3.2 Initial Purification of ABCG2

As the choice of a suitable detergent to solubilize the protein of interest is, among other things, related to the composition of the membrane from which the proteins need to be extracted, 2% OG was used for an initial purification trial of ABCG2. As done with P-gp LPY11-ABCG2 cells were cultured in a fed batch procedure and harvested by centrifugation as described [46]. After mechanical cell disruption and clarification of the lysate, the membrane fraction was collected by ultracentrifugation. ABCG2 was solubilized by 2% OG and thereafter purified using Ni–NTA resin.

Figure 2 shows the results of an ABCG2 purification following solubilization of the crude membranes with 2% OG. ABCG2 eluted from the Ni–NTA as determined by silver staining and immunoblotting (Fig. 2), but the eluate contained only a very small amount of protein. Furthermore other proteins were present in the eluate (Fig. 2a, lane 6), this fact will be referred to at the end of this section. Densitometric analysis of the immunoblot (Fig. 2b) revealed that the 10-histidine tag at the N-terminus of the protein enabled over 90% binding of solubilized protein to the nickel resin, as only a small amount of ABCG2 remained in the flow-through. However, the solubilization itself was only moderately efficient, with 80% of ABCG2 in the starting material present in the unsolubilized fraction. As the eluate already contained 0.4% of mixed lipid stock, purified ABCG2 could be directly reconstituted into a defined lipid environment via dialysis.

It has been previously shown that the presence of specific concentrations of cholesterol has a major impact on ABCG2 function [38, 47]. The average cholesterol content of human MXR cells [38] and MDCKII-BCRP cells [47] both expressing functional ABCG2 is about 23%, therefore a cholesterol concentration of 23% for the resulting ABCG2 proteoliposomes was chosen. The total lipid-to-protein ratio was approximately 270:1 by weight.

Functionality of the purified ABCG2 (R482G) was analyzed by a drug-stimulated ATPase assay using the
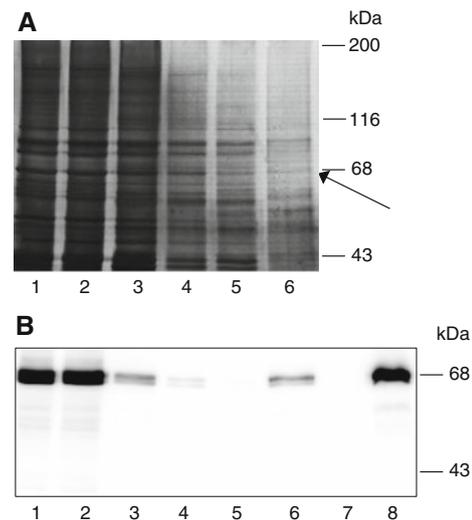
Fig. 2 Purification of ABCG2 from yeast membranes with 2% OG. **a** SDS–PAGE of samples from the purification procedure followed by silver nitrate staining (5 μg per lane for lanes 1–5, 8; 15 μL for lanes 6, 7): from the yeast membranes (lane 1), ABCG2 was solubilized by 2% OG, the insoluble fraction (lane 2) was separated from the solubilized material (lane 3) by ultracentrifugation. The solubilized protein was incubated with Ni–NTA resin and then loaded onto a column. Samples were taken from the flow-through (lane 4) and the washing step (lane 5). After elution from the resin (lane 6) and dialysis, the resulting proteoliposomes were pelleted by ultracentrifugation. **b** Immunoblot analysis of samples from the purification procedure using 2% OG in the same concentration and order as (**a**). Additionally samples from the supernatant after ultracentrifugation of proteoliposomes (lane 7) and of the reconstituted protein (lane 8) are shown

standard stimulator prazosin. 100 μM of this ABCG2 substrate were used in the assay as the strongest stimulation of ATPase activity was described for this concentration [15, 32, 33, 35]. Basal vanadate-sensitive ATPase activity in the absence of added compounds was around 0.18 μmol P_i/min/mg protein. Here, reconstituted ABCG2 was stimulated by 100 μM prazosin roughly twofold (Fig. 3a). This is in consensus with many other published studies using ABCG2-containing membrane vesicles that demonstrated stimulation of ATPase activity by 20 μM prazosin ranging from 1.4 to 2-fold [6, 10, 32] and by 100 μM prazosin ranging from 1.7 to 2-fold [15, 33, 35, 42]. Furthermore, other known substrates such as progesterone and sulfasalazine also stimulated vanadate-sensitive ATPase activity of the purified protein at the concentrations given in Fig. 3a, but to a lesser extent (1.4-fold and 1.5-fold, respectively). This is again similar to the literature [15, 19]. In order to further confirm functionality, a concentration–response curve for ATPase activity utilizing the substrate prazosin was generated. ABCG2 purified using OG was stimulated by prazosin in a concentration-dependent manner with an EC_{50} value of 4.37 μmol/L (Fig. 3b).

A

B
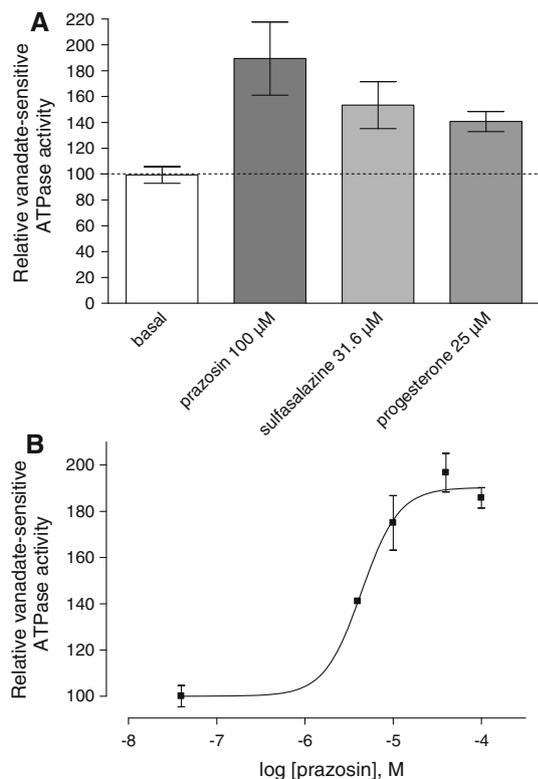
Fig. 3 Assessment of OG-purified ABCG2 functionality by ATPase activity assay. **a** Vanadate-sensitive ATPase activity in the absence of added compounds (basal) and in the presence of the known stimulators prazosin, sulfasalazine, and progesterone. **b** Concentration-effect curve generated with prazosin yielding an EC_{50} value of 4.37 μmol/L. All experiments were performed at least two times in triplicate

3.3 Optimization of the Purification Procedure

OG extracted only a very small amount of ABCG2 from the yeast membranes. To increase the efficiency of the procedure, 15 different detergents were tested for their ability to solubilize ABCG2. The zwitterionic fos-cholines 12–16 (FC-12–16), CHAPS and CHAPSO were tried as well as the non-ionic detergents DM, DDM, triton-X-100, brij 35, brij 58 and cymals 5–7. Only those detergents that demonstrated solubilization are shown in Fig. 4. OG was included in the screen in order to compare it to the initial purification trial, but its efficiency was too low to be detected, as were triton-X-100 and the cymals. The detergents DM, DDM and the fos-cholines solubilized ABCG2 and could be used for purification. Fos-choline 16 (FC-16) extracted the most protein and hence was selected for further screens. Two percent FC-16 solubilized ABCG2 optimally, as determined by immunoblot of the soluble and insoluble fractions (Fig. 5).

Following solubilization trials, purification of ABCG2 from transformed LPY11-ABCG2 (R482G) cells was
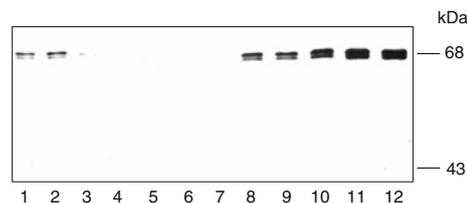
Fig. 4 Detergent solubilization screen for recombinant human ABCG2 from yeast membranes. Membranes containing ABCG2 were solubilized with 1% (w/v) detergent at a total protein concentration of 5 mg/ml (4 μg per lane): DM (lane 1), DDM (lane 2), triton-X-100 (lane 3), OG (lane 4), cymal 5 (lane 5), cymal 6 (lane 6), cymal 7 (lane 7), FC-12 (lane 8), FC-13 (lane 9), FC-14 (lane 10), FC-15 (lane 11), and FC-16 (lane 12). Solubilized samples were subjected to SDS–PAGE (7.5%) and immunoblot analysis

performed with 2% FC-16 as described for OG. SDS–PAGE followed by silver staining (Fig. 6a) and immunoblot analysis of the samples revealed that about 60% of ABCG2 present in the membranes was recovered in the solubilized material (Fig. 6b), which is three times the yield of the OG purification. However, a considerable amount of ABCG2 still remained in the flow-through. The eluted ABCG2 subsequently was reconstituted into proteoliposomes via dialysis.

For the FC-16 purified protein, basal (vanadate-sensitive) ATPase activity could not be stimulated by the addition of 100 μM prazosin (Fig. 7). We concluded that FC-16 was able to solubilize ABCG2 from the yeast membranes with a good yield, but in a form, that cannot be further activated upon reconstitution into proteoliposomes.

For this reason the results from the detergent scan (Fig. 4) were re-analyzed. Besides the fos-cholines 12–16, the non-ionic maltosides DM and DDM solubilized ABCG2 relatively well; however, DDM extracted slightly more protein and was chosen for purification. The optimal concentration for solubilization was determined to be 1.5%
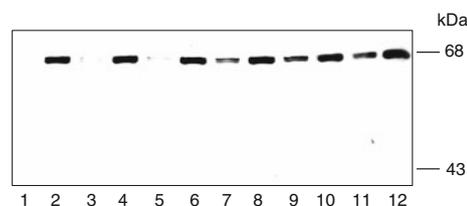
Fig. 5 Determination of the most effective concentration of the detergent fos-choline 16 in solubilization of ABCG2. Samples solubilized with varying percentages of FC-16 at a total protein concentration of 5 mg/ml were analyzed by SDS–PAGE and immunoblotting (4 μg per lane): alternating soluble and insoluble fractions separated by ultracentrifugation, 0% (lanes 1 + 2), 0.5% (lanes 3 + 4), 1% (lanes 5 + 6), 1.5% (lanes 7 + 8), 2% (lanes 9 + 10), and 2.5% (lanes 11 + 12), respectively
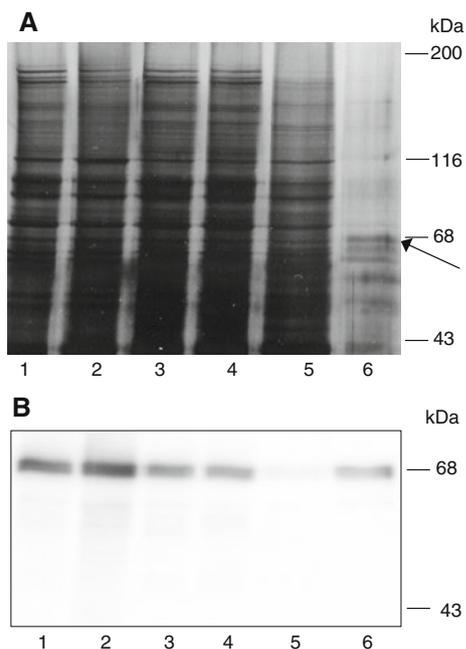
Fig. 6 Purification of ABCG2 from yeast membranes with 2% FC-16. **a** Samples from the purification process were subjected to SDS–PAGE (7.5%) and silver nitrate staining (2 μg per lane for lanes 1–5, 15 μL for lane 6): from the yeast starting material (lane 1), ABCG2 was solubilized by 2% FC-16, insoluble (lane 2) and soluble proteins (lane 3) were separated by ultracentrifugation. The solubilized protein was loaded onto a column after binding to the Ni–NTA. Samples were taken from the flow-through (lane 4) and the washing step (lane 5). The eluate (lane 6) was dialysed and resulting proteoliposomes were collected by ultracentrifugation. **b** Immunoblot analysis of samples (5 μg per lane for lanes 1–5, 15 μL for lane 6) with the same order as (**a**)
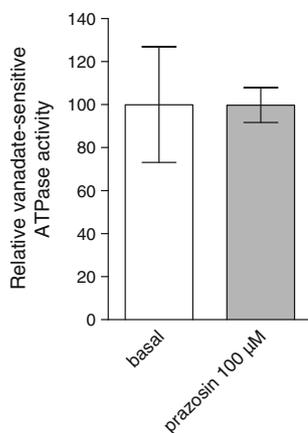
Fig. 7 ATPase activity of ABCG2 purified by 2% FC-16 and reconstituted into liposomes. Vanadate-sensitive ATPase activity in the absence of added compounds (basal) and in the presence of the known stimulator prazosin (100 μM). All experiments were performed at least two times in triplicate

(Fig. 8). Additionally DDM preferably solubilized the upper species of ABCG2, which is hypothesized to be the mature and active form [36].
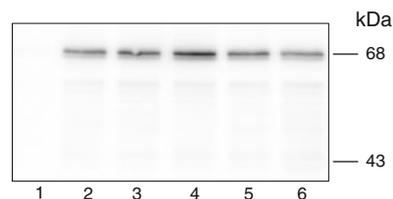
Fig. 8 Determination of the optimal concentration of DDM for ABCG2 solubilization. Samples solubilized with varying percentages of DDM at a total protein concentration of 5 mg/ml were analyzed by immunoblotting (4 μg per lane): soluble fractions of 0% (lane 1), 0.5% (lane 2), 1% (lane 3), 1.5% (lane 4), 2% (lane 5), and 2.5% (lane 6), respectively

ABCG2 was purified using 1.5% DDM as described for OG and FC-16. ABCG2 eluted from the Ni–NTA resin as confirmed by silver staining and immunoblotting (Fig. 9). Densitometric analysis of the immunoblot (Fig. 9b) demonstrated that nearly 50% of ABCG2 in the starting material was solubilized by DDM; however, binding to the Ni-resin was not optimal.

ATPase assays used to investigate the functionality of DDM-purified and reconstituted ABCG2 revealed a basal
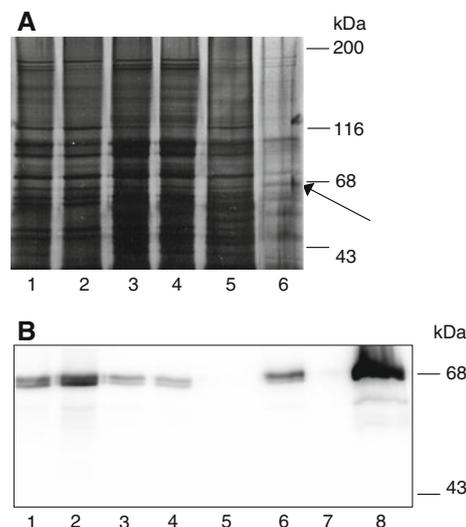
Fig. 9 Purification of ABCG2 from yeast membranes with 1.5% DDM. **a** Samples obtained during the purification were subjected to SDS–PAGE (7.5%) and subsequently stained with silver nitrate (2 μg per lane for lanes 1–5; 15 μL for lane 6): from starting material (lane 1), ABCG2 was solubilized with 1.5% DDM, and ultracentrifugation separated insoluble (lane 2) from soluble fractions (lane 3). Protein bound to the Ni–NTA resin was loaded onto a column, and samples from the flow-through (lane 4) and wash (lane 5) were collected. The protein was eluted (lane 6), dialysed and resulting proteoliposomes were collected. **b** Western blotting of samples (5 μg per lane for lanes 1–5, 8; 15 μL for lanes 6–7) in the same order as in (**a**). A sample was also taken from the supernatant after ultracentrifugation of proteoliposomes (lane 7). Moreover the reconstituted protein is shown (lane 8)
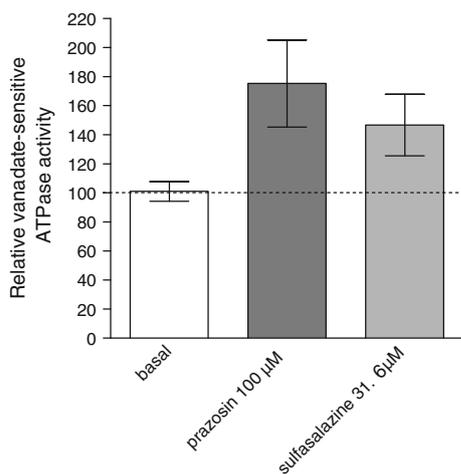
Fig. 10 Assessment of DDM-purified ABCG2 functionality by ATPase activity assay. Vanadate-sensitive ATPase activity in the absence of added compounds (basal) and in the presence of the known stimulators prazosin and sulfasalazine in concentrations depicted. All experiments were performed at least two times in triplicate

vanadate-sensitive ATPase activity of 0.16 μmol P_i/min/mg protein, which is comparable to the basal activity obtained for OG-purified ABCG2. ABCG2 was stimulated by 100 μM prazosin and 31.6 μM sulfasalazine 1.7- and 1.5-fold, respectively (Fig. 10). Furthermore, ATP hydrolysis for this transporter was stimulated by prazosin in a concentration-dependent manner with an EC_{50} value of 4.57 μmol/L. These results indicate that ABCG2 is not only active when purified with 2% OG, but also using 1.5% DDM. An unpaired two-tailed t-test revealed that both the stimulated activities of ABCG2 purified by OG and DDM
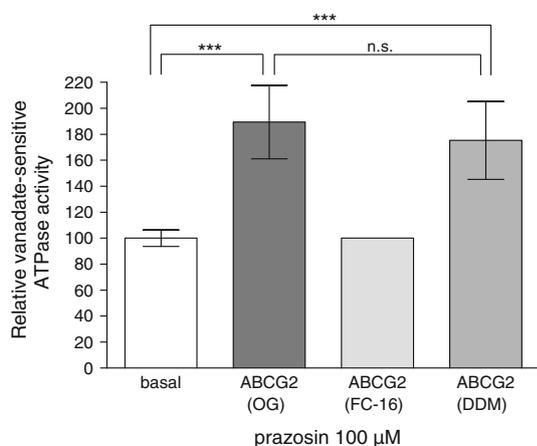
Fig. 11 Comparison of ABCG2 ATPase activity purified by 2% OG, 2% FC-16, and 1.5% DDM, respectively. The data represent vanadate-sensitive ATPase activity in the absence of added compounds (basal) and in the presence of 100 μM prazosin. An unpaired, two-tailed t-test revealed that both the stimulated activities of ABCG2 purified with 2% OG and with 1.5% DDM were significantly higher than basal levels (p values < 0.001). All experiments were performed at least two times in triplicate

(Fig. 11) were significantly increased from the basal activity (p values < 0.001). We assumed a native and active conformation of the purified protein, because its ATPase activity was stimulated by prazosin, progesterone and sulfasalazine. The concentrations required for activation were in agreement with data from the literature for ABCG2 expressed in other organisms, additionally the amount of stimulation was in consensus with the literature [6, 10, 15, 19, 32, 33, 35, 42].

3.4 Directions for Further Experimental Studies

A portion of the solubilized protein in the described purifications with OG or DDM was lost due to failure of binding to the Ni–NTA resin (Fig. 2b, 9b). This was probably a consequence of a protein overload of the resin. Silver staining of samples obtained during the purification processes demonstrated that the eluates from the Ni–NTA resin in both cases were not completely pure (Fig. 2a, 6a and 9a, lane 6).

To investigate if the eluates were contaminated by other yeast proteins or if ABCG2 was proteolytically degraded during the purification process despite the use of a comprehensive protease inhibitor cocktail, these samples were analyzed by immunoblotting using an antibody against the myc epitopes fused to ABCG2 at the N-terminus. This antibody labeled some of the additional proteins (data not shown), therefore these proteins could be degradation products of ABCG2. To further confirm the identity of the most prominent proteins present in the eluates, mass spectrometry analysis was applied. In case of solubilization with OG the principal band and 11 additional bands were analyzed and seven proved to be breakdown products of ABCG2. In case of DDM solubilization showing fewer bands in the SDS–PAGE, all eight bands were analyzed and only one was found to be not ABCG2 or a breakdown fragment. A summary of the identified fragments and their position in the protein is given in Table 1. This observation suggests that to circumvent proteolytic degradation of the purified protein higher concentrations of protease inhibitors or a different combination of these agents should be used during the purification process. In order to further improve the purity of ABCG2, size exclusion chromatography or ultrafiltration steps could also be included into the procedure.

4 Conclusions

A homogenous expression system for the ABC transporter ABCG2 was established in the ergosterol-deficient *S. cerevisiae* strain LPY11. Major advantages over other expression systems such as Sf9 insect cells are the cost-

Table 1 Combined MS fits of OG- and DDM-purified ABCG2 and identified breakdown fragments depicting monoisotopic mass (M + H$^+$), position in the ABCG2 sequence and corresponding amino acid sequence

Monoisotopic mass (M + H$^+$)	Position in ABCG2 R482G	Sequence
675.42	379–383	R.WVSKR.S
801.48	158–163	K.NERINR.V
820.48	178–184	K.VGTQFIR.G
863.48	359–365	K.KKITVFK.E
900.52	466–473	R.VSSYFLGK.L
970.58	324–331	K.QDKPLIEK.L
987.62	315–323	K.ATEIIEPSK.Q
1004.60	418–426	K.NDSTGIQNR.A
1014.62	164–172	R.VIQELGLDK.V
1020.58	48–56	K.LKSGFLPCR.K
1037.54	379–386	R.WVSKRSFK.L
1044.62	87–96	K.SSLLDVLAAR.K
1145.70	148–157	R.LATTMTNHEK.N
1148.64	138–147	R.ENLQFSAALR.L
1172.66	87–97	K.SSLLDVLAARK.D
1231.68	62–72	K.EILSNINGIMK.P
1292.78	252–263	K.LFDSLTLLASGR.L
1296.66	347–358	K.AELHQLSGGEKK.K
1358.74	315–326	K.ATEIIEPSKQDK.P
1360.68	50–61	K.SGFLPCRKPVEK.E
1397.74	161–172	R.INRVIQELGLDK.V
1399.76	617–628	K.QGIDLSPWGLWK.N
1424.68	347–359	K.AELHQLSGGEKKK.K
1433.70	332–343	K.LAEIYVNSSFYK.E
1462.78	178–191	K.VGTQFIRGVSGGER.K
1514.86	164–177	R.VIQELGLDKVADSK.V
1560.68	454–465	K.LFIHEYISGYYR.V
1601.88	48–61	K.LKSGFLPCRKPVEK.E
1791.82	332–346	K.LAEIYVNSSFYKETK.A
1962.91	173–191	K.VADSKVGTQFIRGVSGGER.K
2141.11	347–365	K.AELHQLSGGEKKKKITVFK.E
2247.19	173–193	K.VADSKVGTQFIRGVSGGERKR.T
2253.16	97–118	R.KDPSGLSGDVLINGAPRPANFK.C
2301.22	360–378	K.KITVFKEISYTTSFCHQLR.W
2464.36	62–86	K.EILSNINGIMKPGLNAILGPTGGGK.S
2537.14	231–251	R.MSKQGRTIIFSIHQPRYSIFK.L
2603.23	366–386	K.EISYTTSFCHQLRWVSKRSFK.L
2923.36	148–172	R.LATTMTNHEKNERINRVIQELGLDK.V
2941.39	332–357	K.LAEIYVNSSFYKETKAELHQLSGGEK.K
2957.35	360–383	K.KITVFKEISYTTSFCHQLRWVSKR.S
3194.56	387–417	K.NLLGNPQASIAQIIVTVVLGLVIGAIYFGLK.N
4862.00	315–357	K.ATEIIEPSKQDKPLIEKLAEIYVNSSFYKETKAELHQLSGGEK.K

Masses were matched employing a tryptic in-silico digest using the GPMAW 8.0 software (Lighthouse Data)

effective media and simple culture techniques of yeast. Large quantities of yeast membranes as starting material for purification experiments were easily obtained, and after

performing a screen of 16 detergents for the one that was most efficient at solubilizing ABCG2, it was shown that OG and DDM enabled purification of active protein. The

standard compound prazosin, and other known stimulators of ATPase activity, such as progesterone and sulfasalazine, increased the liberation of inorganic phosphate from ATP. Stimulation of P_i release by the three drugs was in the same range for both ABCG2 purified by OG and DDM. Concentration-effect curves for stimulation of ATPase activity by prazosin could be obtained and for both, OG and DDM, the results were in the same range with an average EC_{50} value of 4.47 μmol/L.

This is the first report of successful reconstitution of human ABCG2 from *S. cerevisiae* membranes retaining ATPase activity, which displays stimulation by prazosin about 2-fold and in a concentration-dependent manner.

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