SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Development of fluorescence methods to study the catalytic cycle of ABC transporters and to determine the ploidy of fish hybrids

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Introduction and literature review

I. ABCG2 and P-glycoprotein

Introduction

The human ABCG2 and P-glycoprotein (Pgp, ABCB1) are primarily active exporter-type ABC transporters that can expel numerous chemically unrelated xeno- and endobiotics from cells, such as chemotherapeutic agents and toxic metabolic products. When expressed in tumour cells (ABCG2, Pgp) or tumour stem cells (ABCG2), they can cause multidrug resistance, often contributing to the failure of chemotherapy. Both proteins are expressed mainly in tissues of the body with barrier functions, including the blood-brain barrier, intestine, liver, placenta, kidney and mammary glands. Due to their tissue localization and broad substrate spectrum, they play an important role in the absorption, distribution, elimination and toxicity (ADME-Tox) of drugs used to treat various diseases. On the other hand, genetic polymorphisms that result in reduced expression and/or impaired function of ABCG2 or ABCB1 may cause altered therapeutic response and drug-related toxicities. As ABCG2 is also a key player in the uric acid elimination pathway, its reduced expression or function may cause hyperuricaemia and gout. They are important drug targets because of their physiological functions and their role in disease pathomechanisms and drug resistance, which requires a detailed understanding of their mechanism of action.

The ABC cassette protein family

The ABC (ATP Binding Casette) proteins are one of the most abundant protein families, with members found in all living organisms from bacteria to humans. They share a high degree of conservation of ATP-binding site structure and similarity in ATP binding and utilization. Their ATP binding sites are characterised by the presence of three conserved sequence motifs: the Walker A sequence (GXXGXGKS, where X can represent any amino acid), the Walker B sequence (hhhhD, where h represents a hydrophobic amino acid) and the "ABC-signature" (LSGGQQ/R/KQR) motif, which is unique to ABC proteins.

ABC transporters are generally composed of two structurally and functionally distinct domains: the globular nucleotide-binding domain (NBD) located in the cytoplasm, which is the catalytic centre of the protein, where ATP is bound and hydrolysed, providing the energy for substrate transport; and the membrane-embedded transmembrane domain (TMD), which forms the substrate-binding sites and substrate translocation pathway.

TMDs are usually composed of 6 membrane-spanning alpha helices. A functional ABC protein is usually composed of at least two NBDs and two TMDs, since the two ATP-binding sites are formed jointly by the NBDs.

The structure of ABCG2 and Pgp

Based on the crystal structure of many ABC transporters and biochemical and biophysical experiments, it is likely that eukaryotic ABC transporters adopt similar conformational states during their function. These are the 'inward facing' (IF) conformation open to the intracellular space and the 'outward facing' (OF) conformation open to the extracellular space. Under ATP-free conditions, the two NBDs dissociate, giving rise to the IF TMD conformation, in which ABCG2 and Pgp form an inverted "V" shape with a large cavity in the middle, where the substrate-binding pocket of the proteins is accessible to substrates from both the inner membrane layer and the intracellular space. Following nucleotide binding, dimerization of the two NBDs creates an OF conformer that is closed towards the intracellular space but open towards the extracellular space, responsible for the removal of substrates to the extracellular space.

High-resolution cryo-EM (cryo-electronmicroscopy) structures of ABCG2 and Pgp confirmed the conformational changes expected from the crystal structure of other ABC transporters. However, it is important to note that the conformers identified by the cryo-EM technique represent only snapshots of the complete catalytic cycle.

The catalytic cycle of ABCG2 and Pgp

The major events in the transport process by ABCG2 and Pgp are the binding of substrates, their translocation across the plasma membrane and finally their dissociation from the binding site to the extracellular space. Substrate transport is associated with conformational changes of TMDs, which is regulated by ATP-dependent formation and dissociation of the NBD "sandwich dimer". Our previous results suggest that dimerization of NBDs during the Pgp functional cycle is triggered by binding of ATP molecules, resulting in an extracellularly open state of TMDs. These conformational changes are also accompanied by a decrease in substrate affinity at substrate binding sites, which promotes the export of substrates against a concentration gradient. However, in the case of ABCG2, it is still unclear how nucleotide binding and hydrolysis are related to conformational changes in TMDs and whether ATP binding or hydrolysis triggers conformational changes in the transporter during which substrates are dissociated from substrate binding sites created by transmembrane domains that are open to the extracellular space.

The driving force of the catalytic cycle is ATP hydrolysis, which can occur at either ATP binding site for both transporters, but it is still unclear how many ATP hydrolysis events are required to transport a substrate molecule. It is a commonly observed phenomenon for some ABC transporters that their different substrates enhance the ATPase activity of the transporters in a structure- and concentration-dependent manner, but it is not known which catalytic step is used to accelerate this effect. Interestingly, even in the absence of their substrates, a weaker ATPase activity, termed basal ATPase activity, can be observed.

It is generally accepted that both ATP-binding sites must be catalytically active for ABC transporters to function. In the case of Pgp, it has also been observed that inactivation of one of the catalytic sites (e.g., by mutations) prevents ATPase activity in Pgp. However, in contrast to these results, our group has previously shown that human Pgp molecules exhibit

significant transport and ATPase activity when one of the NBD Walker A lysines is replaced by methionine, when tested in the plasma membrane of mammalian cells in their natural environment. It is important to highlight that the majority of studies reporting that mutations generated at one of the catalytic sites also have an inactivating effect have been performed using heterologous expression systems (e.g., Sf9 or Saccharomyces cerevisiae) or purified proteins. Since it is likely that the previous data are not relevant to the function and cooperation of NBDs, it is worth repeating the functional study of mutant transporter variants in the plasma membrane of mammalian cells.

Observing changes in ABCG2 and Pgp conformation

There are antibodies that recognise complex epitopes, such as MRK16, MRK17, HYB-241, 4E3, 15D3 and UIC2 for Pgp and 5D3 for ABCG2. MRK16, HYB-241 and UIC2, which recognise Pgp, are also known to inhibit transport activity of Pgp.

UIC2 is an IgG2a isotype mouse monoclonal antibody (mAb) that selectively recognises the IF conformer of human Pgp with high substrate binding affinity. The complex epitope of the antibody is formed by short peptide sequences in extracellular loops 1, 4 and 6 of human Pgp, which are spaced apart in the OF conformer.

In intact cells, UIC2 can bind to only about 10-40% of cell surface Pgp molecules when applied at saturating concentrations, but in the presence of certain substrates or inhibitors of the protein, the remaining Pgp molecules can be detected. This phenomenon is often referred to as the "UIC2 shift" in the literature. Among others, such Pgp inhibitor is cyclosporin A (CsA), known for its immunosuppressive effect. In addition to treatment with Pgp inhibitors, ATP depletion of cells also results in UIC2-reactive conformation of Pgp's. However, addition of ATP/Mg2+ to cells deprived of ATP by permeabilization and washing reduces UIC2 reactivity in a concentration-dependent manner.

Like UIC2, the complex extracellular epitope-binding 5D3 recognizes ABCG2 in a conformationally sensitive manner. The extracellular loop (EL3) connecting TM (transmembrane) helices 5 and 6 is involved in the formation of the conformational epitope. Cryo-EM studies suggest that the antibody recognises the nucleotide-free IF conformation of the protein, whereas in the OF conformation the extracellular loops forming the epitope are presumably distant from each other, and thus the antibody cannot bind to this conformer.

Although it recognizes all cell surface ABCG2 molecules when administered at saturating concentrations, its reactivity at lower concentrations depends on the actual conformation of the protein. ATP depletion of cells or pre-treatment with ABCG2 inhibitors (e.g., Ko143) enhances 5D3 binding. In contrast, treatment with phosphate analogues (e.g., vanadate (Vi), or beryllium fluoride (BeFx)), which trap ABCG2 molecules in the post-hydrolytic state, significantly reduces 5D3 binding.

Effect of membrane environment on the function of multidrug transporters

Transmembrane proteins, such as ABCG2 and Pgp, are in close contact with the surrounding membrane, whose physicochemical parameters have a significant impact on the

function of the proteins. The properties of the membrane can affect the transporters' conformational changes, their transport activity, the distribution of their substrates in the lipid bilayer or even their interaction with the transporters' substrate binding sites. Cholesterol is a major constituent of mammalian plasma membranes. Certain microdomains such as lipid rafts and caveolae are cholesterol-rich membrane regions. ABCG2 as well as Pgp have been identified in cholesterol-rich lipid rafts in several cell types. Reducing the cholesterol content of the membrane significantly decreases the transport activity of both proteins, whereas high cholesterol content of the membrane enhances the ATPase and transport activity of ABCG2. It is likely that interactions with membrane cholesterol and phospholipid molecules are crucial for the activity of Pgp and ABCG2. Recently, it has been described for ABCG2 that cholesterol (which is not a substrate for ABCG2) inhibits the entry of phospholipids into the substrate-binding pocket that can bind to the substrate site, which may explain the increased transport activity associated with higher membrane cholesterol content.

In addition to the specific lipid composition of the membrane, its physicochemical properties may also influence the function of membrane proteins. The substrates ABCG2 and Pgp are mostly hydrophobic molecules whose rate of passive diffusion across the cell membrane is highly dependent on the physicochemical properties of the membrane.

The function of ABCG2 and Pgp has been studied in various experimental systems, e.g. lipid-detergent micelles, artificial lipid bilayers. It is possible that these experimental systems do not adequately mimic the physiological membrane environment and may lead to conflicting results due to differences in their physicochemical properties.

Since the function of Pgp and ABCG2 is strongly influenced by their membrane environment, model systems where transporters can be studied in their natural membrane environment are of crucial importance.

II. Determination of ploidy of fish red blood cells and embryos by high-throughput flow cytometry

The importance of triploid individuals in aquaculture industry

Triploid fishes are generally sterile, as the irregular meiotic segregation of chromosomes reduces gonadal development and induces abnormal gametogenesis. It is economically important to produce sterile triploid individuals because they require less energy for reproductive processes, resulting in faster growth rates and longer life spans in many species. Interestingly, the glycogen and fat content of triploid fish can also differ, affecting their nutritional value and taste. It is important to note that the establishment of sterile triploid individuals in pond farms can prevent the spread of alien species outside the farms, thus avoiding the genetic and/or ecological impact of escapes on natural fish populations. Sterile triploids of alien fish species can even be released into natural waters for aquatic weed control without the risk of overpopulation or the introduction and spread of undesirable species.

Biotechnological methods, such as pressure shock, heat shock or cold shock are used to create triploid offsprings. The biological basis of these procedures is that the shock induces the retention of a polar body during the second meiotic division and therefore, the triploid

offspring inherits two maternal and one paternal chromosome sets. Unfortunately, triploid induction treatments are not 100% effective, so determining the proportion of triploid larvae produced during a given treatment can help researchers to optimise their protocols.

Sturgeons and their hybridisation

Our collaboration partners wanted to breed female specimens collected in the framework of the Danube genetic conservation programme for Danube sturgeon using meiotic ginogenesis to preserve their favourable genetic make-up. During ginogenesis, the semen only initiates embryo development, so it is preferable to use semen from a species with which the sturgeon cannot hybridise. Since no successful hybridisation has been described between the families Acipenseridae and Polyodontidae, the sperm of the American paddlefish was used to induce ginogenesis. However, the phenotypic appearance of the resulting progeny raised the possibility that hybridization of the two phylogenetically distant species did occur. Therefore, to shed light on the origin of the progenies we performed flow cytomtery-based DNA content analysis.

Determination of DNA content by flow cytometry

The determination of DNA content by flow cytometry using a suitable DNA-specific fluorescent dye is a simple but sensitive technique. Other methods, such as chromosome number determination, are more labour intensive and require the presence of actively dividing cells. Flow cytometric DNA content determination is also frequently used in taxonomic and evolutionary studies to determine the ploidy of plants and various animal species. In addition to research applications, ploidy determination is of increasing importance in crop and animal production, as well as in fish farming.

However, despite its simplicity, the method can have numerous pitfalls. Inadequate fixation and permeabilisation of samples may cause aggregation or disintegration of cells, leading to false results or even prevent the analysis of samples. The use of membrane-impermeable DNA-specific dyes necessitates permeabilisation of cells. However, the choice of reagents used to permeabilize and/or fix the cells may also affect the stoichiometric staining of the nuclear DNA. Unfortunately, in fisheries, the analysis of samples cannot always be performed on site and therefore, there is a great need for sample fixation and storage methods that can be easily implemented under field conditions and do not degrade the quality of samples. In addition, there is a need to develop simple experimental protocols that allow rapid preparation and analysis of large number of samples.

Aims of study

I. Despite the fact that the catalytic cycle of ABCG2 has been intensively studied for a long time, it is still unclear how the processes of ATP binding and cleavage are related to TMD conformational changes and how they ultimately allow the transport of substrates against their concentration gradient. Considering the sensitivity of ABC transporters to the composition of their membrane environment, we aimed to develop experimental methods to study the catalytic cycle of ABCG2 in its natural membrane environment. In our experiments, we aimed to answer the following questions:

- Which catalytic event triggers the transition from the 5D3-reactive IF conformation to the 5D3-dim OF conformation?
- How does the substrate affinity of ABCG2 change during the catalytic cycle?
- How is the turnover rate of ATP hydrolysis accelerated by substrates in ABCG2?

II. In the second part of our work, we investigated the function of Pgp variants carrying unilateral or bilateral point mutations in the A-loop (Y401A, Y1044A, Y401A/Y1044A) or Walker B sequence motif (E556M/Q, E1201Q, E556Q/E1201Q, D555N, D1200N, D555N/D1200N) to gain insight into the cooperation between the two NBDs. In our experiments, we aimed to answer the following questions:

- How different ligands affect the the fraction of the UIC2-reactive IF conformer in different mutant variants?
- Whether the mutations alter the apparent ATP affinity (K_A) of Pgp?
- Are the mutants capable of substrate-dependent ATP hydrolysis?

III. When optimising triploid induction treatments in fish farms, it is important to quickly and accurately determine the proportion of triploid individuals. Therefore, we wanted to develop a flow cytometric experimental protocol for the determination of ploidy in fish red blood cells and fish embryos, which allows the rapid preparation and analysis of large numbers of samples.

Materials and methods

Cell culture

The MDCK II (Madin-Darby canine kidney) cell line and its stably transfected version with ABCG2 were provided by Balázs Sarkadi (Research Centre for Natural Sciences, Institute of Enzymology, Budapest, Hungary). The MDCK II cell line expressing ABCG2 labelled with GFP (Green Fuorescent Protein) was prepared by László Homolya and Tamás Orbán (Research Centre for Natural Sciences, Institute of Enzymology, Budapest, Hungary). The NIH 3T3 mouse fibroblast cell line and its stably transfected version with the human MDR1 gene (NIH 3T3 MDR1 cell line) were obtained from the laboratory of Michael Gottesman (National Institutes of Health, Bethesda, MD, USA). Transgenic NIH 3T3 cell lines expressing loop A (Y401A, Y1044A) and Walker B (D555N, D1200N, D555N/D1200N, E556M, E556Q, E1201Q, E556Q/E1201Q) mutant Pgp variants were generated by Dóra Türk and Gergely Szakács (Research Centre for Natural Sciences, Institute of Enzymology, Budapest, Hungary) using a Sleeping Beauty transposon-based gene expression system.

Cells were maintained in scanning cultures in 10% heat-inactivated fetal bovine serum (FBS, Gibco, Budapest), DMEM (Dulbecco's modified Eagle's medium) containing 2 mM L-glutamine and 0.1 mg/mL penicillin-streptomycin cocktail at 37°C in a 5% CO2 atmosphere at 95% humidity. For cells expressing wild-type human Pgp, high Pgp expression levels were maintained by 670 nM doxorubicin (DOX) selection. Cells were transferred to DOX-free medium 2 days before experiments were performed.

NIH 3T3 mouse fibroblast cells were separated from the bottom of the cell culture flask and from each other by 0.05% trypsin-EDTA solution (0.5 mg/mL trypsin, 0.2 mg/mL EDTA, 2 min, 37°C), and MDCK II cells were separated from the bottom of the cell culture flask and from each other by 0.25% trypsin-EDTA solution (2.5 mg/mL trypsin, 0.2 mg/mL EDTA, 5 min, 37°C).

Western-blot analysis

Cells (2×10^5 cells/mL) were lysed in 100 µl reducing Laemmli sample buffer (6×) for 10 min at 95 °C. Afterward, the lysates were subjected to SDS-polyacrylamide gel electrophoresis using an 8% polyacrylamide gel and then electroblotted onto a nitrocellulose membrane with a pore size of 0.45 µm (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK). ABCG2 expression was detected by the BXP-21 mouse mAb, while actin was labeled with the C-2 mouse mAb (both from Santa Cruz Biotechnology Inc, Santa Cruz Biotechnology, CA). As a secondary antibody, a goat anti-mouse HRP-conjugated IgG (Santa Cruz Biotechnology Inc, Santa Cruz, CA) was applied. All antibodies were used at 1:2500 dilution. Bands were visualized with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA) using the FluorChem Q gel documentation system (Alpha Innotech Corp, San Leandro, CA).

Mitoxantrone accumulation assay

The transport activity of ABCG2 and ABCG2-GFP was studied using an MX accumulation assay. Cells (0.5×10^6 cells/mL in PBS containing 7 mM glucose [gl-PBS]) were pre-incubated in the presence or absence of 2 μM Ko143 for 15 min at 37°C and then stained with 5 μM MX for 30 min. Samples were washed three times with ice-cold gl-PBS containing 0.5% fetal bovine serum (FBS) and stored on ice until flow cytometry measurement. To exclude dead cells from the analysis, samples were stained with propidium iodide (PI).

Direct immunofluorescent labelling of ABCG2

Cells (0.5×10^6 cells/mL in gl-PBS) were pre-incubated with or without 2 μM Ko143 for 10 min and then further incubated with 5 μg/mL 5D3-A647 monoclonal anti-ABCG2 antibody at 37°C. After 30 min of incubation, samples were washed two times with ice-cold gl-PBS and centrifuged for 5 min at 435×*g* at 4°C. The 5D3-A647 fluorescence intensity of the cells was measured by flow cytometry.

Determination of the proportion of Pgp molecules in the reactive conformation of UIC2

Intact NIH 3T3 cells (5×10^5 cells/mL) expressing wild-type and mutant Pgp variants were labeled with UIC2-A647 (10 μg/mL) or 15D3-A647 (30 μg/mL) antibody in gl-PBS for 30 min at 37°C. CsA-treated samples were preincubated with 10 μM CsA for 10 min at 37°C and further incubated with UIC2-A647 antibody without washing step. ATP-depleted samples were treated with Na-azide (10 mM) and 2-deoxy-D-glucose (8 mM) for 30 min at 37°C in glucose-free PBS prior to antibody labeling. After incubation with antibodies, cells were washed twice with ice-cold gl-PBS containing 0.5% FBS. For ATP-depleted samples, glucose-free PBS was used. UIC2 reactivity, i.e. the percentage of cell surface Pgp molecules in the UIC2-reactive conformation, was calculated from the ratio of D/P-corrected UIC2 and 15D3 signals.

Cell permeabilisation with Streptolysin-O toxin

Streptolysin-O ((SLO) (Sigma-Aldrich, Budapest, Hungary) is a pore-forming exotoxin of Streptococcus pyogenes. SLO pores formed in the membrane are permeable to small water-soluble molecules, including nucleotides. SLO is an oxygen-sensitive toxin that is reversibly activated by dithiothreitol (DTT). Cells (1×10^7 cells/mL) were treated with 250 U/mL SLO in the presence of 1 mM DTT, Protease Inhibitor Cocktail (PIC: (2 mM AEBSF, 0.3 μM aprotinin, 116 μM bestatin, 14 μM E1-14 μM leupeptin), 0.5 mM phenylmethylsulphonyl fluoride (PMSF) and 1% FBS in gl-PBS at 37°C for 30 min. Under these conditions, approximately 50% of the cells were permeabilized, as confirmed by PI staining. The reaction was stopped with 20 mL of PBS containing 1% FBS, and then the cells

were washed three times (635×g, 5 min, room temperature) to remove unbound toxin, and finally the samples were taken up in PBS for further treatments.

For confocal microscopy experiments, cells grown on eight-chamber coverslips (ibidi GmbH, Gräfelfing, Germany) were permeabilized with 62.5 U/mL SLO in the presence of 1 mM DTT and PIC at 37°C for 15 min in HEPES buffer containing 1% FBS (20 mM HEPES, 123 mM NaCl, 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM $CaCl_2$).

To investigate the kinetics of 5D3 antibody binding, permeabilized cells (1×10^6 cells/mL) were stained with 5 μg/mL 5D3-A647 antibody in the absence or presence of 0.5 mM ATP/Mg^{2+}, 2 μM Ko143 and 10 μM quercetin at 37°C. To follow the kinetics of 5D3-A647 binding, samples were taken at different time points and washed with high volume ice-cold PBS. To remove unbound antibody, samples were washed twice more with ice-cold PBS. The extent of 5D3-A647 binding was determined using a FACS Array (Becton Dickinson, Mountain View, CA, USA) flow cytometer.

Determination of the apparent affinity of nucleotide binding

Apparent affinity of nucleotide binding (K_A) was determined as described previously. Permeabilized cells (1×10^6 cells/mL) were pre-treated with nucleotides added at different concentrations in the presence of equimolar concentrations of Mg^{2+} at 37°C for 10 min and then further incubated with 5 μg/mL 5D3-A647 at 37°C for 20 min. To prevent ATP hydrolysis, ATP was added without Mg^{2+} in the presence of 5 mM EDTA or the whole experiment was carried out on ice. In nucleotide trapping experiments, nucleotide treatments were applied together with 0.5 mM Vi or BeFx (200 μM $BeSO_4$ and 1 mM NaF) at 37°C for 30 min. Subsequently, the cells were labeled with 5 μg/mL 5D3-A647 on ice for 45 min after removal of the un-trapped nucleotides by washing them two times with ice-cold PBS. After antibody labeling samples were washed again three times with ice-cold PBS and centrifuged for 5 min at 635×g at 4°C. The mean 5D3-A647 fluorescence intensity of the cells was determined by flow cytometry and plotted as a function of the nucleotide concentration. To determine the apparent affinity of ABCG2 for nucleotides (K_A), data points were fitted with the four-parameter Hill function, where the F_{min} and F_{max} values represent the minimum and maximum fluorescence intensities, respectively:

$$F = \frac{F_{min} \times K_A^n + F_{max} \times x^n}{K_A^n + x^n} \quad (1)$$

Nucleotide trapping

Permeabilized cells (1×10^6 ml^{-1}) were incubated with 0.5 mM ATP/Mg^{2+} or ADP/Mg^{2+} and 0.5 mM V_i or BeF_x in the presence or absence of ABCG2 substrates (10 μM quercetin or 10 μM estrone-3-sulfate (E1S)) in PBS at 37°C. To follow the kinetics of the trapping reaction, 500 μL aliquots was taken at different time points and washed two times with 5 ml ice-cold PBS. After washing, the samples were resuspended in 500 μL ice-cold PBS and labeled with 5 μg/mL 5D3-A647 at 4°C for 45 min. The 5D3-A647 fluorescence intensity of the samples (F) was plotted as a function of time (t). The $t_{1/2}$ values, representing the half-

life of the 5D3-reactive ABCG2 conformation, were calculated from an exponential fit of the data points according to the following equation:

$$F = F_0 \times e^{-t \times \frac{ln2}{t_{\frac{1}{2}}}} + c \quad (2)$$

Wherein F_0 is the difference between the zero and infinite time points of the curve and c is the background fluorescence intensity of cells.

5D3 dissociation

Permeabilized MDCK-ABCG2 cells (1×10^6 cells/mL) were pre-labeled with 5D3-A647 in the presence or absence of 10 μM quercetin or 10 μM E1S for 20 min at 37°C. After removing the unbound 5D3-A647, cells (1×10^5 cells/mL) were further incubated with 3 mM ATP/Mg^{2+} or AMP-PNP/Mg^{2+} in the absence or presence of the above substrates at 37°C. To study the kinetics of 5D3 dissociation, 500 μL aliquots were taken at regular intervals and washed two times with ice-cold PBS. The 5D3-A647 fluorescence intensity of the cells was measured by flow cytometry and plotted as a function of time (t). The $t_{1/2}$ values, representing the half-life of the 5D3-reactive ABCG2 conformation, were calculated from an exponential fit of the data points using Equation 2.

Flow cytometry

The fluorescence intensity of cells was measured using a Becton Dickinson FACS Array flow cytometer (Becton Dickinson, Mountain View, CA) and the data were evaluated using Flowing software (Cell Imaging Core, Turku Centre for Biotechnology, Turku, Finland). The A647 dye was excited with the 635 nm laser and fluorescence was detected in the red channel (emission 661/16 nm). The PI dye was excited with the 532 nm laser and emission was detected with a 585/42 nm bandpass filter. MX was excited with a 635 nm laser and fluorescence was detected in the far red channel (emission: 780/60 nm). Cell debris and clumped cells were excluded from the analysis based on FSC and SSC signals. For studies on intact cells, the PI-negative cell population was considered, whereas for studies with permeabilized cells, the PI-positive cell population was analysed.

Confocal laser scanning microscopy and fluorescence co-localization analysis

To assess the co-localization of ABCG2-GFP and the fluorescent ABCG2 substrate MX in the plasma membrane of MDCK cells, we carried out confocal laser scanning microscopy (CLSM) experiments. Measurements were performed in eight-well chambered coverslip plates (ibidi GmbH, Gräfelfing, Germany). ATP depletion of intact cells was induced by a 15 min pre-treatment with 8 mM 2-deoxy-D-glucose and 10 mM sodium azide in glucose-free medium. ATP depleted or non-ATP depleted cells were pre-treated with 2 μM Ko143 or 0.5 mM Vi for 15 min, stained with 500 nM MX for 15 min at 37°C and then

washed three times with HEPES solution. SLO-permeabilized cells were pre-stained with 6 μg/ml PI, then further incubated with 500 nM MX for 15 min at 37°C in the presence or absence of 5 mM AMP-PNP and subsequently washed three times with HEPES solution.

Fluorescence images were acquired with a Nikon A1 Eclipse Ti2 Confocal Laser-Scanning Microscope (Nikon, Tokyo, Japan) using a Plan Apo 60× water objective [NA=1.27]. Laser lines of 488 nm and 647 nm were used for the excitation of ABCG2-GFP and MX, while fluorescence emissions were detected through band pass filters of 500-550 nm and 660-740 nm, respectively. All the images were recorded with the same settings of the equipment, such as same high voltages, laser powers and pinhole. Images were acquired in sequential mode to minimize the crosstalk between channels. Images of approximately 1 μm thick optical sections, each with 512×512 pixels, and a pixel size of approximately 200 nm, were acquired. A spatial averaging filter with a 3×3 mask was used to denoise the images. Co-localization analysis was carried out by calculating the Pearson's correlation coefficients between the pixel intensities of the two detection channels in pixels representing the plasma membrane. Only pixels where at least one of the intensities was above the threshold (2× the average auto fluorescence intensity) were included in the analysis. Image analysis methods and routines were implemented in MATLAB scripts (Mathworks Inc., Natick, MA).

Fluorescence correlation spectroscopy (FCS)

To distinguish free and ABCG2-bound MX molecules based on their different diffusion properties, fluorescence correlation spectroscopy (FCS) measurements were performed. FCS measurements were carried out using a Nikon A1 Eclipse Ti2 Confocal Laser-Scanning Microscope (Nikon, Tokyo, Japan), equipped with a Plan Apo 60× water objective [NA=1.27] and a PicoQuant time-correlated single photon counting FCS (TCSPC-FCS) upgrade kit (PicoQuant, Berlin, Germany).

FCS measurements were carried out on live MDCK cells expressing ABCG2-GFP in eight-well chambered coverslip plates (ibidi GmbH, Gräfelfing, Germany). Cells were stained with 100 nM MX for 15 min at 37°C in the presence or absence of 2 μM Ko143 or after ATP depletion. Fluorescence of ABCG2-GFP and MX was excited with a 488 nm and a 647 nm laser, respectively. The fluorescence signals emitted by ABCG2-GFP and MX were detected in the spectral ranges of 500-550 nm and 660-740 nm using single photon counting detectors (PicoQuant, Berlin, Germany). Measurements of 10×10 second runs were taken at three selected points in the cross-section of the plasma membrane of each selected cell. Fluorescence autocorrelation curves were calculated using SymPhoTime64 software (PicoQuant, Berlin, Germany) at 200 time points from 300 ns to 1 s with a quasi-logarithmic time scale.

Autocorrelation curves of the doubly labeled cells were fitted to a triplet state model with two diffusion components to describe the 3D-diffusion of free MX (fast component) and the 2D diffusion of ABCG2-bound MX in the x-z plane of the plasma membrane (slow component). The laser beam was positioned in a region of the cell membrane parallel to the long axis of the ellipsoidal laser volume.

$$G(\tau) = \frac{1-T+Te^{\frac{-\tau}{\tau_{trip}}}}{N(1-T)}\left(\rho\frac{1}{1+\frac{\tau}{\tau_{D1}}}\frac{1}{\sqrt{1+\frac{\tau}{S^2\tau_{D1}}}} + (1-\rho)\frac{1}{\sqrt{1+\frac{\tau}{\tau_{D2}}}}\frac{1}{\sqrt{1+\frac{\tau}{S^2\tau_{D2}}}}\right) \quad (3)$$

In equation (3), N is the average number of fluorescent molecules in the detection volume, T is the fraction of molecules in the triplet state, τ_{trip} is the triplet correlation time. The diffusion rate is characterized by the diffusion time τ_D, which is the average time spent by a molecule in the illuminated volume. τ_{D1} and τ_{D2} are the diffusion times of the fast and slow components, ρ is the fraction of the first component and 1-ρ is the fraction of the second component. The diffusion coefficients (D) of the fast and slow components were determined from the following equation:

$$D = \frac{\omega_{xy}^2}{4\tau_d} \quad (4)$$

Wherein, ω_{xy} is the lateral e^{-2}radius of the detection volume. ω_{xy} was measured by determining the diffusion time of 100 nM A647 dye (dissolved in 10 mM Tris, 0.1 mM EDTA-containing buffer, pH 7.4) with known diffusion coefficient (D_{A647} = 330 $\mu m^2/s$, at T = 22.5°C) and substituting it into Equation 4 that corresponds to the aspect ratio of the ellipsoidal confocal volume, defined as the ratio of its axial and radial dimensions. This parameter was estimated by fitting the autocorrelation curves of a 100 nM A647 dye solution.

Membrane preparation

For ATPase activity measurements, membrane preparations from NIH 3T3 cells expressing wild-type or mutant Pgp variants were used. Cell membranes were isolated from NIH 3T3 cells by differential centrifugation. Cell debris and nuclei were sedimented at 500×g for 10 min at 4°C. Membrane fractions were isolated by centrifugation at 12000×g for 60 min at 4°C. Membrane pellets were resuspended in TMEP solution (50 mM Tris, pH=7.0, HCl) supplemented with 50 mM mannitol, 2 mM EGTA, 0.5 mM phenylmethylsulphonyl fluoride (PMSF) and protease inhibitor cocktail (Sigma-Aldrich, Budapest). Protein concentrations of membrane samples were determined by Lowry's method. Membrane samples were stored at -80°C until use. The transporter expression (5 μg membrane protein/sample) of membrane samples was checked by immunoblotting with anti-Pgp G-1 monoclonal antibody (Santa Cruz Biotechnology Inc. CA, USA) and horseradish peroxidase (HRP)-labeled secondary antibody (Santa Cruz Biotechnology Inc. CA, USA) at 1:5000 dilution.

ATPase activity measurement

The Vi-sensitive ATPase activity of wild-type and mutant Pgp was determined by colorimetric assay. The specific ATPase activity of the transporters was calculated from the amount of Pi released. For the substrate-stimulated ATPase activity assay, membrane preparations were incubated in the presence or absence of Pgp substrate verapamil (40 μM) and 3 mM ATP/Mg^{2+} for 25 min at 37°C. The reaction was then stopped with 40 μL of 5% SDS and the samples were incubated for 30 min at room temperature with 105 μL of color

reagent. The absorbance of the samples was measured at 700 nm using a BioTek Synergy HT plate reader (BioTek Instruments, Winooski, VT, USA).

DNA content analysis

The DNA content of red blood cells (RBC) and fish embryos was determined by flow cytometry after PI staining. A cell suspension of individual cells was prepared from fish embryos by treatment with 100 µg/mL Proteinase K and 5 mM EGTA (15 min at 37°C). After washing (500×g, 5 min, 4°C), cells were resuspended in 500 µL gl-PBS (10^7 cells/mL). Blood cells were centrifuged (500×g, 5 min) and resuspended in 500 µL gl-PBS to adjust the cell concentration to 1×10^7 cells/mL. Then, 4500 µL of 70% ethanol (Scharlab Hungary, Budapest) was added to the samples and the cells were fixed and permeabilized for 30 min at room temperature. Samples were then washed twice in PBS (500×g for 5 min and resuspended in 5000 µL PBS. At this time, ethanol-fixed cells were stained with 40 µg/mL PI (Sigma-Aldrich) for 30 min, and the PI fluorescence of the cells was measured using a Becton Dickinson FACS Array flow cytometer.

Statistical analysis and curve fitting

For the statistical analysis of data, SigmaPlot (version 14, SSI San Jose, CA, USA) was used. For the comparison of two samples from normally distributed populations with equal variances, Student's *t*-test was performed, while in case of unequal variances a Kolmogorov–Smirnov test was applied. Multiple comparisons were performed with analysis of variance (ANOVA) applying the Holm–Sidak test for *post hoc* pair-wise comparison of the data. In the case of unequal variances, the Dunnett T3 *post hoc* pair-wise comparison method was used. Differences were considered significant at $p < 0.05$.

All curve fitting was carried out by SigmaPlot (version 14, SSI San Jose, CA, USA) except for fitting of autocorrelation curves that was performed by using the QuickFit 3.0 software developed in the group B040 (Prof. Jörg Langowski) at the German Cancer Research Center (DKFZ).

Results

I. The catalytic cycle of ABCG2

MDCK II cells express fully functional ABCG2 and ABCG2-GFP

ABCG2 and its N-terminally GFP-tagged variant (ABCG2-GFP) were expressed at comparable levels in MDCK II cells. In accordance with literature data, the transport activity of ABCG2 was not influenced by the GFP-tag. Previous observations showed that ABCG2 inhibitors, such as Ko143 can enhance 5D3 mAb binding by shifting the equilibrium to the IF state. Accordingly, in the plasma membrane of untreated live cells ABCG2-GFP and ABCG2 exhibited comparably low 5D3-reactivity, which was increased in a similar extent by Ko143 treatment, supporting the notion that the GFP-tag does not modify the conformational response of ABCG2.

MDCK II cells can be permeabilized with streptolysin-O

Considering the sensitivity of ABC transporters to the composition of their membrane environment, we wanted to develop an experimental system that would allow the study of the catalytic cycle of ABCG2 in the plasma membrane of normal cells. We wanted to investigate the concentration-dependent effects of different nucleotides on changes in ABCG2 conformation. Since nucleotides do not cross the intact plasma membrane due to their negative charges, cells must be carefully permeabilized. MDCK II cells are well permeabilized with the pore-forming Streptolysin O (SLO) toxin from Streptococcus pyogenes. The toxin binds to the membrane cholesterol and oligomerises to form a permeable pore for small molecules.

Cells were treated with SLO applied at different concentrations, and the percentage of permeabilized cells was determined by PI staining in a flow cytometer. Since SLO is oxygen labile and thus the efficiency of permeabilization is reduced in oxidative environments, its effect on cells was also investigated in the presence of a reducing agent (DTT, dithiothreitol). At high SLO concentrations, a significant fraction of cells were permeabilized, and this was further enhanced by the presence of DTT. However, we also observed that the reactivity of 5D3 to the ABCG2 protein was reduced at high DTT concentrations, presumably because DTT reduces the disulfide bridges involved in ABCG2 homodimer formation. To avoid structural changes in the ABCG2 protein, 1 mM DTT was used in further experiments. After permeabilization, nucleotides were removed from the cells by washing and then loaded with nucleotides or nucleotide analogues (e.g., ATP, ADP, AMP-PNP) of the desired type and concentration.

Nucleotide binding is sufficient to trigger the switch from the 5D3-reactive IF conformation to a 5D3-dim OF conformation

To study the nucleotide dependent conformation changes of ABCG2, we systematically changed the intracellular nucleotide concentrations in semi-permeabilized cells. In accordance with an ATP-regulated switch of the TMD conformation, increasing ATP/Mg^{2+} concentrations gradually decreased the 5D3-A647-reactivity of ABCG2-positive cells, with practically zero staining at high ATP/Mg^{2+} concentrations. To prevent nucleotide hydrolysis, ATP was either added in the absence of Mg^{2+}, on ice, or ATP was replaced with the non-hydrolysable ATP analogue AMP-PNP. Interestingly, the conformational change driving ABCG2 into a 5D3-dim (i.e., 5D3 non-reactive) state also occurred in the absence of ATP hydrolysis and showed similar nucleotide concentration dependence. These results indicate that the 5D3-dim and 5D3-reactive conformations correspond to the OF and IF conformations as observed in ATP-bound and nucleotide-free crystal structures, respectively.

By replacing the cleaved gamma phosphate following ATP hydrolysis, phosphate analogues, such as Vi or BeFx, trap ABC transporters in a stable ternary complex (ABCG2-ADP-Vi/BeFx). Based on different geometries of myosin structures obtained with transition state analogs, the BeFx- or Vi-trapped post-hydrolytic complexes are believed to represent pre- and post-hydrolytic conformations, respectively. Co-treatment with Vi or BeFx increased the apparent nucleotide affinity of ABCG2 about 10-fold in conditions permitting ATP hydrolysis, confirming that both phosphate analogues can form stable ADP-trapped complexes with ABCG2. When ATP hydrolysis was prevented, the phosphate analogues did not have any effect on the K_A values.

Similar to the bacterial ABC transporter MsbA ADP/Mg^{2+} could also induce the IF to OF switch, albeit at slightly higher concentrations (K_A = 7.38 ± 2.31 mM) compared to ATP/Mg^{2+}. Moreover, nucleotide trapping occurred in the presence of phosphate analogs and ADP/Mg^{2+}. The K_A values obtained with ADP/Mg^{2+} and Vi (K_A = 0.44 ± 0.1 mM) or BeFx (K_A = 0.57 ± 0.32 mM) did not differ from the K_A values of trapping reactions starting from ATP/Mg^{2+}. However, in the trapping reactions with ADP/Mg^{2+}, about 30% of ABCG2 molecules remained in a 5D3-reactive state even in the presence of very high nucleotide concentrations. This observation may suggest that the ternary complex resulting from ADP/Mg^{2+} is different (i.e., less stable, possessing a shorter lifetime compared to the complex produced from ATP/Mg^{2+} in the hydrolytic cycle) and therefore the trapping reaction occurs with lower efficiency. Since in energized cells the cytosolic ATP concentration is more than 10-fold higher compared to ADP concentrations, these results indicate that in live cells, (i) the switch from the 5D3-reactive IF to a 5D3-dim OF conformation is induced by ATP binding; and (ii) resetting to the 5D3-reactive IF conformation can only occur after release of the hydrolysis products.

Substrates increase the rate of formation of the Vi- or BeFx-trapped species

Transported substrates increase the turnover rate of ATP hydrolysis in many ABC transporters including ABCB1 and ABCG2. Progressive accumulation of the transporter molecules in the stable Vi- or BeF_x-trapped post-hydrolytic states represents a partial reaction

of the catalytic cycle in ABC transporters. Accordingly, the accumulation of ABCG2 in the Vi- or BeFx-trapped post-hydrolytic complex was accelerated by substrates. Estrone-3-sulphate (E3S) and quercetin induced about a 5-fold increase in the rate of the trapping reaction (for $t_{1/2}$ values), which is consistent with the extent of stimulations achieved by these compounds in the ATPase assay.

The relatively long $t_{1/2}$ values compared to the total cycle time, which is in the order of 100 ms (as inferred from ATPase data), suggest that formation of a stable post-hydrolysis complex by phosphate mimicking anions is a low-probability event. The increased rate of the trapping reaction in the presence of substrates may be explained by a higher turnover of the ATPase cycle, or a longer duration of the Vi- or BeFx-"sensitive" state, that is between the dissociation of the cleaved phosphate and the disassembly of the NBD dimer. Consistently, trapping reactions starting from ADP/Mg^{2+} were not accelerated by quercetin or E3S, suggesting that substrates do not affect the overall stability or lifespan of the ADP-bound, phosphate analogue-sensitive ABCG2 conformer. This can happen, if substrates similarly accelerate the formation and the dissociation of the ADP-bound conformer, or alternatively, they do not have any effect on these processes.

ABCG2 ligands do not affect 5D3 binding kinetics

We next examined the effects of ATP/Mg^{2+}, quercetin and Ko143 on the time kinetics of 5D3 mAb binding. We found that approximately 20-25 min were required for maximal binding of 5D3 mAb. In the presence of ATP/Mg^{2+} and the substrate quercetin, the transporters showed decreased 5D3 reactivity, whereas the ABCG2 inhibitor Ko143 significantly increased 5D3 binding of the protein. Interestingly, antibody binding kinetics were not affected by ABCG2 ligands. Thus, we concluded that by examining the kinetics of antibody binding, we cannot draw conclusions regarding the kinetics of transporter conformation changes.

Substrates accelerate the IF to OF transition of ABCG2

With the aim to pinpoint the transition that is accelerated by transported substrates, in the following experiments we studied how nucleotides and substrates affect the kinetics of the IF to OF transition detected by a shift in 5D3 binding. To align ABCG2 molecules in an IF state, semi-permeabilized (nucleotide-free) MDCK cells expressing ABCG2 were pre-labeled with 5D3-A647 antibody. Unbound 5D3-A647 molecules were removed, and cells were incubated at 37 °C in a sufficiently large volume to prevent rebinding of the antibody. Under these conditions, we observed a gradual decrease of the 5D3-A647 fluorescence of cells, which was completely prevented by Ko143 treatment, supporting the notion that the ABCG2 molecules are intrinsically dynamic, while Ko143 stabilizes them in the IF 5D3-reactive conformation. The dissociation rate of the antibody was significantly enhanced in the presence of transported substrates or ATP/Mg^{2+}. However, the largest (about 5-fold) decrease of the $t_{1/2}$ values corresponding to the half-life of the 5D3-bound ABCG2 conformer was observed when substrates were co-administered with ATP/Mg^{2+}.

In further experiments, intracellular nucleotide pools were replenished with a non-hydrolysable ATP analogue AMP-PNP/Mg^{2+}. In the absence of ATP hydrolysis, ABCG2 molecules undergo IF to OF transition, and the backward transition to the IF state has an extremely low probability. The time dependence of the AMP-PNP/Mg^{2+}-induced 5D3 dissociation was comparable to that obtained with ATP/Mg^{2+} supporting that the antibody dissociation kinetics observed either in the presence of ATP/Mg^{2+} or AMP-PNP/Mg^{2+} may reflect the first nucleotide-induced IF to OF transition of ABCG2. Strikingly, when co-administered with AMP-PNP/Mg^{2+}, the same substrates did not increase further the dissociation rate of 5D3, suggesting that the NBD dimer formation induced by AMP-PNP/Mg^{2+} binding switches ABCG2 into the low drug binding affinity state. However, similar decrease of the $t_{1/2}$ values was observed when substrates were added before AMP-PNP/Mg^{2+} treatment, indicating that transition to the OF state can also be accelerated by substrates with AMP-PNP/Mg^{2+} treatment. Collectively, the above data imply that substrate binding at the TMDs induces a structural change in the transporter that can facilitate the nucleotide-dependent NBD dimer formation and the concomitant IF to OF transition probably by reducing the energy barrier of the above conformational changes.

The nucleotide-free IF conformation of ABCG2 has higher substrate affinity compared to the Vi-trapped post-hydrolytic conformation

In the following experiments, we visualized the subcellular localization of ABCG2-GFP and the fluorescent ABCG2 substrate MX by using confocal microscopy. In accordance with previous observations, at low concentrations, MX only stained poorly the MDCK ABCG2-GFP cells, while ATP depletion, Ko143 or Vi treatments increased the intracellular accumulation of MX. Interestingly, ATP depleted cells exhibited strong plasma membrane staining by MX. Plasma membrane staining by MX in both native and ATP depleted cells was abolished by treatment with the competitive inhibitor Ko143, suggesting that red fluorescence in the plasma membrane of ATP-depleted cells reflects MX binding to ABCG2 molecules. To quantify the fraction of MX-bound ABCG2 molecules, we calculated the Pearson's correlation coefficients (PCC) between the MX and ABCG2-GFP signals in pixels representing the plasma membrane. Since the ABCG2-GFP signal was unchanged during the course of the different treatments, the correlation coefficients depend mostly on MX binding to the transporter. In ATP depleted cells, the high correlation values indicate that the majority of ABCG2 molecules reside in a MX-bound conformation (PCC = 0.72 ± 0.12). The correlation between the two signals strongly decreased in the presence of Ko143 (PCC = -0.1 ± 0.18), suggesting displacement of MX from the substrate binding site of the transporter by the competitive inhibitor. Similar results were obtained when ATP depletion was combined with Ko143 treatment (PCC = -0.04 ± 0.17). Binding of MX to ABCG2 was also suppressed by Vi (PCC = 0.18 ± 0.13), which is consistent with the notion that the post-hydrolytic ABCG2 conformer possesses low substrate affinity. Interestingly, in untreated cells, we measured significantly higher co-localization between the MX and ABCG2-GFP signals (PCC = 0.3 ± 0.12) than in Ko143-treated cells, suggesting that in the plasma membrane of live cells, a significant subset of ABCG2 molecules resides in an MX-bound IF state.

MX binding to ABCG2 is confirmed by its reduced mobility using FCS measurements

As an independent approach to follow MX binding to ABCG2, we measured the mobility of MX in the plasma membrane by fluorescence correlation spectroscopy (FCS). MX molecules bound to ABCG2 are expected to show decreased diffusion compared to free MX. We analyzed the fluorescence autocorrelation functions (ACFs) of MX and ABCG2-GFP in the plasma membrane using a two-component model. Upon ATP-depletion, the diffusion coefficient of MX decreased to the level obtained for ABCG2-GFP, indicating that MX molecules readily bind to the nucleotide-free IF conformer of ABCG2. In accordance with the data obtained from cellular distributions, the competitive inhibitor Ko143 prevented MX binding to ABCG2, resulting in the dominance of a high plasma membrane mobility MX population similar to the only-MX treated cells.

AMP-PNP binding switches ABCG2 to a conformation that is unable to bind MX

Permeabilization of cells with SLO synchronizes ABCG2 molecules in a nucleotide-free, 5D3-reactive IF conformation also observed in cryo-EM structures. When permeabilized cells were treated with MX alone, we measured a strong co-localization between ABCG2-GFP and MX in the plasma membrane (PCC = 0.85±0.05), confirming the high substrate affinity of the IF conformation of ABCG2. Strikingly, pre-incubation of permeabilized cells with 5 mM AMP-PNP/Mg^{2+} strongly reduced the co-localization between MX and ABCG2-GFP in the plasma membrane (PCC = -0.12 ± 0.20), indicating that the conformational changes induced by AMP-PNP binding prevented MX binding to ABCG2.

II. Functional analysis of NBD mutant Pgp variants

A-loop and Walker B mutations affect the fraction of UIC2-reactive IF Pgp conformers

In further experiments, we investigated whether mutations in the conserved sequences of one or both NBDs affect the proportion of UIC2-reactive IF Pgp conformers. In untreated intact cells, approximately 20% of wild-type Pgp were UIC2-reactive IF conformers. ATP depletion of cells and the competitive Pgp inhibitor CsA trapped nearly 100% of cell-surface Pgp molecules in an ATP-free UIC2-reactive IF state, consistent with literature data. Interestingly, only about 5% of the E556M and E556Q mutant proteins were in the UIC2-reactive conformation, whereas the ATP-binding-weakening A-loop mutants had a significantly increased proportion of UIC2-reactive conformers compared to the wild-type transporter. Similarly, increased UIC2 reactivity was observed in D555N and D1200N mutants. Interestingly, ATP depletion of cells or CsA treatment switched all mutant variants to the UIC2-reactive IF conformation by nearly 100%. Under the influence of the phosphate analogue Vi, wild-type proteins are trapped in the ATP hydrolyzed state, which our group has

previously shown to be in the UIC2-unbinding OF state. Upon Vi treatment, we observed a significant decrease in the proportion of IF conformers in both the half-site Walker A and Walker B aspartate mutants, suggesting that at least the intact catalytic center is capable of ATP hydrolysis in these variants. In the variants that are likely to bind ATP with high affinity (E556M, E556Q), Vi treatment did not cause a further decrease in the proportion of IF conformers.

NBD mutations affect nucleotide binding affinity

Nucleotide binding to NBD induces conformational changes in transporter TMD regions due to allosteric coupling. We systematically altered intracellular ATP/Mg^{2+} concentrations in cells permeabilized with SLO toxin. Consistent with ATP-regulated changes in the conformation of TMDs, increasing ATP/Mg^{2+} concentrations resulted in decreased UIC2-A647 labeling. For wild-type Pgp, the apparent affinity of ATP/Mg^{2+} (K_A=1.56 ± 0.46 mM) was increased by approximately one order of magnitude by Vi treatment. Compared to wild-type Pgp, E556M and E556Q variants (K_A=0.23 ± 0.06) showed lower K_A values in both the absence and presence of Vi, indicating that the exchange of 'catalytic glutamate' results in tight nucleotide binding.

An ATP-dependent decrease in UIC2 reactivity was also observed for the half-side A-loop mutant Y401A and Y1044A, but the K_A values obtained indicate a reduced ATP affinity compared to the wild-type protein. The decrease in K_A values upon Vi treatment makes it likely that Y401A, Y1044A, E556M and E556Q variants are able to hydrolyze ATP at least at their intact catalytic sites. For the other mutant transporter variants (Y401A/Y1044A, D555N, D1200N, D555N/D1200N), we observed very weak ATP affinity values (10 mM<), which did not change significantly upon Vi treatment, suggesting that these proteins are not capable of ATP binding and hydrolysis.

Pgp molecules carrying Y401A and E556M mutations show measurable ATP hydrolysis activity

We further investigated whether the Pgp variants (Y401A, E556M, E556Q), which are presumably ATP hydrolysis competent based on our previous experiments, exhibit measurable ATPase activity. To this end, we measured the Pgp-dependent steady-state ATPase activity of membrane preparations prepared from NIH 3T3 cells expressing wild-type or similarly high levels of different mutant Pgp variants. Compared to Pgp-negative preparations, we measured significantly higher basal ATPase activity in preparations expressing wild-type Pgp (approximately 2 mmol Pi/mg protein/min), which was increased approximately 3-fold by the Pgp substrate verapamil (40 μM). Although extremely low basal ATPase activity was observed in Y401A and E556Q mutants, the activity of both mutants was enhanced by substrate administration. However, the ATPase activity of preparations expressing the E556M variant did not differ from that of Pgp-negative samples. These results support the ability of Y401A and E556Q mutants to hydrolyze ATP in repeated cycles.

III. Determination of ploidy of fish red blood cells and embryos by high-throughput flow cytometry

Diploid and triploid fish red blood cells can be discriminated by flow cytometry

A flow cytometry-based method applying on stoichiometric (proportional to DNA content) binding of PI DNA dye was established to determine the ploidy of fish red blood cells. To optimize the method, cell fixation was also tested by treatment with 95% ethanol and 70% ethanol. In the analysis of Amur erythrocytes, cells showed an increased tendency to aggregate when 95% ethanol fixation solution was used, so 70% ethanol solution was used for further analysis. Diploid Amur red blood cells were used as controls. PI fluorescence intensity and light scattering parameters of 20,000 cells per sample were determined to ensure a statistically adequate number of elements. We found that some of the samples showed fluorescence intensities about one and a half times higher than the known diploid samples. The method has been validated on other fish species, e.g., carp (*Cyprinus carpio*), perch (*Perca fluviatilis*), which gave similar results. The Forward Scatter (FSC) parameter proportional to cell size was also investigated. For cells with higher PI fluorescence, we detected a FSC signal about one and a half times larger compared to diploid samples, which correlated with an increase in PI fluorescence intensity.

The size of fish red blood cells is proportional to their ploidy level

In the following, the size of diploid and triploid amur red blood cells was also investigated in a Bürker chamber using a light microscope. The area of the red blood cells was determined from the digitised images using Image J software. Consistent with our flow cytometric PI fluorescence intensity and FSC measurements, the cell area in triploid samples was significantly larger than that of cells from diploid controls.

DNA analysis of amur embryos

Successful blood sampling requires individuals to reach a certain size, which in the case of large populations results in significant additional costs for fish farms, so in further experiments we have developed a method to determine the DNA content of amur embryos at a few days of age. For this purpose, we prepared cell suspensions of individual cells from a few days old individuals by proteinase digestion and determined the DNA content of the cells by PI staining. The unknown samples were compared to the DNA content of diploid control amur embryos. Compared to the diploid control sample, the sample from the probable triploid individual showed a fluorescence intensity about one and a half times higher.

Determination of DNA content of hybrids derived from unintentional crosses between Russian sturgeon and American paddlefish

Flow cytometry measurements were performed on 98 hybrid fish, two sturgeon (6F15, Ag03) and four American paddlefish (2E75, 551F, 596B, 6517). All measurements were repeated three times independently. Blood samples were analysed by the blind method, except for the four male American paddlefish. The average PI fluorescence intensity of the hybrid individuals was normalized to the average of the PI fluorescence intensities of the three male American paddlefish and the DNA content was expressed in pg according to the modified formula of Tiersch et al. DNA content (pg) = 3.9× (I/P), where I is the PI fluorescence intensity of the hybrids and P is the PI fluorescence intensity of the paternal individuals. The constant 3.9 is the DNA content of the paddlefish expressed as a picogram.

Based on the DNA content analysis, two main groups of hybrid individuals were distinguished: a small genome size, presumably triploid group (SH) with a DNA content of 7.22 ± 0.71 pg, and a large genome size, presumably pentaploid group (LH) with a DNA content of 11.60 ± 0.84 pg. Karyotype analysis was also performed by our collaborators on some samples with different DNA content.

Discussion

I. The catalytic cycle of ABCG2

According to the alternating access model formulated by Jardetzky more than 50 years ago, membrane transporters alternate between IF and OF states, in which the centrally located substrate-binding site is accessible to only one side of the membrane at a time. In active transporters, such as ABC transporters, accessibility changes are accompanied by a significant change of substrate affinity that is linked to binding and hydrolysis of ATP. Based on homology models and cryo-EM structures, ABCG2 is believed to alternate between a nucleotide-free IF and a nucleotide-bound OF conformation during its transport cycle. The switch between the NBD-dissociated IF and the NBD-associated OF states involves a series of conformational changes that finally result in the uphill transport of substrates. In agreement with cryo-EM studies, we show in nucleotide titration experiments that 5D3 exclusively recognizes the nucleotide-free IF conformation of ABCG2, as the 5D3 recognized epitope of ABCG2 only forms in the IF conformation, while it is disrupted in the OF conformation.

While semi-permeabilized cells provide a unique tool to study ABCG2 in a quasi-natural environment, the antibody-shift assay has several limitations. Because of the conformation selectivity of 5D3, details of the allosteric coupling between the NBDs and TMDs are inferred based on the influence of the population of ABCG2 molecules in the IF state, without any direct information on the intermediate or OF sates. Still, by modulating the levels of various nucleotide species, distinct steps of the ATPase cycle, such as nucleotide binding or ATP hydrolysis. Moreover, we characterized MX binding to ABCG2 to better understand the crosstalk between nucleotide and substrate binding. While the interactions of fluorescent substrates with purified ABC transporters have been studied in nanodiscs or styrene maleic acid lipid copolymer particles, to our knowledge we have applied the FCS technique for the first time to study substrate binding to ABCG2 in its natural plasma membrane environment in live cells.

In accordance with structural studies, our confocal microscopy experiments carried out in live cells show that the *apo* form of ABCG2 possess high MX-affinity, while nucleotide binding and the concomitant dimerization of the NBDs induce conformation changes that prevent MX-binding. The simultaneous drop in the 5D3- and MX-binding indicates that the high-to-low switch in drug binding affinity coincides with the transition from the IF to the OF conformation. Ko143, which can displace MX from the substrate binding site also prevents the transition of ABCG2 from the IF to the OF conformation in agreement with previous cryo-EM studies. Our data also directly indicate that high affinity binding of MX requires the IF conformation and that energizing the transporter by nucleotides is not required for substrate binding. The nucleotide titration experiments prove that nucleotide binding (ATP, AMP-PNP and ADP) and concomitant NBD dimer formation is sufficient to induce the conformational switch to the OF conformation, and that ATP hydrolysis is not essential for the nucleotide-dependent IF-OF transition and the high-to-low switch in drug binding affinity, supporting the results of structural studies carried out using the catalytic glutamate mutant ABCG2 variant. We also observed that in the presence of physiological ATP concentrations, in the context of the plasma membrane of live cells, a small, but significant fraction of ABCG2 molecules

reside in the MX-bound state ready to initiate a productive transport cycle. The V_i-trapped post-hydrolysis state possesses low substrate affinity, confirming that dissociation of the hydrolysis products is required to switch the transporter from the OF conformation back to the IF conformation and to reset its high substrate affinity.

Previous studies observed analogous changes of the substrate binding affinity in ABCB1, ABCC1 and ABCG2 using radio ligand binding assays. ABCB1 exists in an equilibrium between OF and IF states, which are readily interconverted by ligand binding. A similar, bidirectional interdomain cross-talk between the NBDs and TMDs was observed in human ABCB1 using the UIC2-reactivity assay. The fact that the antibody binding site and the NBDs of the transporters are ~80 Å apart and on opposite sides of the membrane suggests that long range conformational couplings between the TMD and NBD motions are conserved features among human ABC exporters. However, the similarity in coupling of the ATPase activity and the transport cycles of ABCB1 and ABCG2 is remarkable in view of the differences in the conformational changes of the TMDs and the substrate binding cavities, and the different modes of the nucleotide dependent NBD motions.

Like several other ABC transporters, ABCG2 possesses significant basal ATPase activity, which is generally increased 2 to 5-fold by transported substrates. The increased catalytic turnover is reflected by the enhanced steady-state ATP hydrolysis rate and the increased rate at which ABCG2 becomes trapped in the Vi- or BeFx-bound post-hydrolytic states.

Our experiments demonstrated that 5D3 binding to ABCG2 is reversible, and addition of substrates, ATP/Mg^{2+} or their combination can accelerate the kinetics of antibody dissociation. The reversible nature of 5D3 binding also explains previous observations that treatments with saturating concentration of the antibody did not induce complete inhibition of transport and of ATPase activity.

Importantly, 5D3 dissociation experiments carried out in the absence of nucleotides clearly showed that substrate binding alone has an effect on the conformation of ABCG2. When substrates are presented prior to the nucleotides, the IF to OF transition is accelerated. In contrast, when the nucleotide analogue AMP-PNP is allowed to bind first, substrates cannot promote the transition. Thus, substrates accelerate the cumulative step of nucleotide binding, NBD "sandwich-dimer" formation and the concomitant IF to OF transition, but only when having access to the substrate binding site as present in the IF state. The substrate induced conformational change might be small as indicated by a recent study, which could not detect these changes as an increase in the proximity ratio of FRET between the NBDs of ABCB1 in permeabilized cells deprived of ATP/Mg^{2+}. Accordingly, in recent cryo-EM studies carried out at turnover conditions (in the presence of substrate and ATP/Mg^{2+}) two conformers representing the transition from the IF state to a semi-closed state were identified. These structures revealed that the accessibility of the substrate binding site gradually decreases upon the closure of the NBD dimer, and therefore, substrates should bind to the IF conformer.

In conclusion, our results indicate that nucleotide binding is the major regulator of TMD conformation in ABCG2. The nucleotide induced IF to OF transition coincides with the high-to-low switch of substrate affinity, and this event precedes ATP hydrolysis. Detailed kinetic analysis of several ABC transporters will be needed to establish differences and

similarities of the catalytic mechanisms within the superfamily. In the case of the channel-type ABC protein CFTR (ABCC7), pore opening, believed to correspond to the IF to OF transition, represents the slowest step of the gating cycle. In contrast, in the case of ABCC1 and ABCB1, the IF to OF transition is not the rate-limiting step of the catalytic cycle. Similarly, during determination of the apparent affinity of nucleotide binding we experienced that in the presence of saturating concentrations of hydrolysable ATP, the majority of ABCG2 molecules adopt the 5D3-dim OF conformation, indicating that the IF to OF transition is not the slowest step of the catalytic cycle and consequently it is not the major determinant of the cycle time. Accordingly, in live cells, only a small fraction of ABCG2 molecules is in the substrate-bound IF conformation ready to harvest the energy of ATP for transport, while depletion of ATP increases the proportion of ABCG2 molecules in the IF conformation by prolonging the waiting time before the IF to OF transition. How can we explain the stimulation of the steady-state ABCG2 ATPase activity by transported substrates? On the one hand, our 5D3 dissotiation measurements clearly demonstrate that bound substrates accelerate the IF to OF transition, probably by facilitating the nucleotide-dependent NBD dimer formation. However, a mere speeding of the IF to OF transition cannot significantly increase the turnover rate, suggesting that substrate-mediated stimulation of the ATPase must reflect acceleration of some other step(s). At the same time, our confocal microscopy results indicate that substrates can no longer bind to the OF, low-substrate-affinity conformation, and therefore their influence on the rate constants of ATP hydrolysis and the concomitant NBD dissociation can be ruled out. This apparent paradox may be resolved by assuming two distinct pre-hydrolytic OF states associated with an uncoupled, basal activity and a coupled, drug transport dependent ATPase activity. In the coupled activity pathway, transported substrates increase the catalytic rate of ABCG2 by accelerating the IF-OF transition similar to ABCC1. In the absence of drugs, the transporter follows a different catalytic path, in which the step limiting the uncoupled ATPase activity follows the IF-OF transition. Eventually, ABCG2 molecules that have reached the NBD sandwich dimer without substrate binding either dissociate without hydrolysis, or undergo a futile ATP hydrolysis cycle to reset the transporter into an IF state with high drug binding affinity. In the future, single molecule-based approaches may provide further insights into the kinetics of the above transitions.

II. Functional analysis of NBD mutant Pgp variants

Similar to ABCG2, Pgp undergoes ATP binding- and hydrolysis-dependent conformational changes, which ultimately allows transport of substrates against their concentration gradient. Pgp has two canonical, functionally equivalent ATP-binding sites, each of which can bind and hydrolyse ATP, providing the energy requirement for the catalytic cycle. However, it is not known how the ATP hydrolysis is coordinated between the two NBDs and whether Pgp hydrolyses one or two (or more) ATP molecules in one duty cycle. It is generally believed that the integrity of both catalytic centres is required for transport, as previous studies have shown that inactivation of one NBD results in complete inhibition of ATPase and transport activity alike.

Our results confirmed that bilateral mutation of A-loop tyrosines (Y401A/Y1044A) or Walker B aspartates (D555N/D1200N) stabilized the Pgp molecules in the UIC2-reactive IF conformation. However, similar to wild-type Pgp, addition of ATP/Mg^{2+} induced the IF-OF transition in unilateral A-loop (Y401A and Y1044A) mutant Pgp variants. Similar to wild-type Pgp, Vi increased the apparent affinity for nucleotide binding in the unilateral A-loop mutants. Since Vi-dependent trapping is an extremely low probability event, the formation of a stable Vi-trapped complex and the significant left shift of the UIC2 binding curves support that the mutant transporter variants are also capable of ATP hydrolysis in repeated cycles. Accordingly, we also observed significant substrate-stimulated ATPase activity in the unilateral A-loop tyrosine mutants. Previously our research group made analogous observations with unilateral Walker A mutant Pgp variants. These data collectively support that one intact NBD is sufficient for the catalytic activity of Pgp. Assuming a strict alternation of ATP hydrolysis between the two binding sites, every second ATP hydrolysis event would occur at the inactive binding site, resulting in inhibition of the catalytic activity. Nevertheless, we have observed reduced but significant ATPase activity for certain unilateral NBD mutants (e.g., Y401A, E556Q), supporting the random engagement of the catalytic centres for ATP hydrolysis.

It is known from the literature that ABC transporters are highly sensitive to their membrane microenvironment, so changes in membrane properties can affect their function. Interestingly, previous studies reporting the inactivating effect of unilateral Walker A and A-loop mutations used heterologous expression systems (e.g., *Sf9* or *Saccharomyces cerevisiae* cells) and/or purified proteins. In contrast, our group studied different Pgp variants in their natural plasma membrane environment, in intact or permeabilized mammalian cells. The plasma membranes of insect cells and yeast cells contain less cholesterol compared to mammalian cells, which may explain the different results.

In my experiments, unilateral Walker B aspartate mutants (D555N or D1201N) were also able to switch to the UIC2-dim OF conformation, but this occurred at ATP concentrations much higher than the physiological intracellular ATP level. My work is continued by new members of the research group. Consistent with my results, they also could not detect transport activity of Walker B aspartate mutants in intact cells, and did not observe significant ATPase activity at the physiological 3 mM ATP concentration. However, future ATPase measurements carried out at higher ATP concentrations may also confirm the functional activity of Walker B aspartate mutants.

III. Determination of ploidy of fish red blood cells and embryos by high-throughput flow cytometry

Propidium iodide (PI) is an intercalating DNA-specific dye, which binds to the DNA stoichiometrically, so the measured fluorescence intensity is proportional to the DNA content of cells. Since PI is not a membrane permeable dye, cells were fixed and permeabilized before PI staining. Our staining protocol followed by flow cytometry-based evaluation of the cellular DNA content was found to be sensitive enough to distinguish between cells with diploid and triploid chromosome sets. Fluorescence intensities and light scattering parameters of 20,000

cells per sample were determined to obtain statistically adequate data. Using a Becton Dickinson FACS Array cytometer we could analyse around 100 samples in 1 to 1.5 hours making possible high-throughput measurements. The Forward scatter (FSC) parameter is directly proportional to cell size, allowing different cell sizes to be distinguished. It is known from literature data that the size of red blood cells from artificially created amur triploid hybrids is significantly larger than that of diploid amur red blood cells, so in addition to determining DNA content, we also examined the FSC signal of cells. We found that triploid cells identified on the basis of their DNA content exhibited about 1.5-fold higher FSC value compared to the diploid control cells. This conclusion was also confirmed by light microscopy measurements. Since the size difference between triploid and diploid red blood cells was found to be easily recognisable to the trained eye, microscopic examination can be performed in fish farms where expensive flow cytometers are not available.

Next, we used the PI staining method to determine the DNA content of hybrid fish embryos obtained from unintentional crosses between Russian sturgeon and American paddlefish. We have shown that despite the large phylogenetic distance, crosses between female sturgeon and male American paddlefish resulted in viable hybrids with fertilization, hatching and survival rates very similar to those of the parent species. By measuring the DNA content of red blood cells we have identified both triploid and pentaploid individuals among the hybrid offsprings. Our studies were confirmed by our collaborator applying morphological analysis, karyotyping and microsatellite DNA determination. Within the triploid and pentaploid groups a slight variation in chromosome number was observed during karyotyping, which may be due to inaccuracy in the determination of the microchromosome number or may be caused by the loss of microchromosomes, which is common in interspecies hybrids with different ploidy levels.

Overall, with the development of high-throughput flow cytometers and appropriate sample preparation and storage protocols, flow cytometry-based DNA content analysis and ploidy determination could become a very useful routine method in the hands of researchers working in aquaculture.

Summary of results

I. In the first part of our work, we attempted to gain a better understanding of the catalytic cycle of the wild-type human ABCG2. We have developed fluorescence-based methods that allowed us to study the transporter in its natural membrane environment and made the following observations:

- ATP binding is sufficient to switch ABCG2 from the 5D3-reactive IF to the 5D3-dim OF conformation.
- The decrease in substrate affinity coincides with the switch of the transporter to the OF state.
- Substrate binding promotes the IF-OF transition, whereas certain ABCG2 inhibitors such as Ko143 prevent this conformational change.
- Since nucleotide binding is the main regulator of the TMD conformation, for a productive transport cycle substrate binding should precede ATP binding.
- The hydrolysis of ATP and the subsequent dissociation of the hydrolysis products allows ABCG2 to revert to its high substrate affinity IF conformation to initiate a new transport cycle.

II. In the second part of our work we investigated the conformational changes and the ATPase activity of unilateral and bilateral A-loop and Walker B mutant Pgp variants with the following conclusions:

- Unilateral A-loop (Y401A, Y1044A) and Walker B (E556Q) mutant Pgp variants can be trapped in the post-hydrolytic state either in intact or permeabilized cells and accordingly they are capable of significant ATP hydrolytic activity.
- Our results further promote that Pgp variants with a single intact catalytic site are catalytically active.

III. In the last part of our work, to improve the efficacy of triploid induction treatments applied in aquaculture industry, we tested the ploidy level of fish red blood cells and fish embrios. In these studies we have optimized a simple PI-staining-based flow cytometry assay in various fish species.

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