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

Studying conformational changes and membrane microdomain localization of P-glycoprotein

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The examination took place at Discussion Room, Institute of Immunology, Faculty of Medicine, University of Debrecen at 14.30 pm 23th May 2014.

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The PhD Defense takes place at the Lecture Hall of Building A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen at 13.00 pm 13th January 2020.

1. Introduction

Resistance to classical chemotherapeutic agents and novel targeted drugs remains a major problem in cancer therapy despite the intense development of cancer treatment protocols during the past decades. Cellular drug resistance is responsible for treatment failure in 90% of patients with metastatic cancer. Drug resistance can be classified into two categories: intrinsic (primary) resistance is present before starting any chemotherapy, while acquired (secondary) resistance is induced during the course of chemotherapy by the applied drug(s). Although the development of drug resistance is induced by a single chemotherapeutic agent or a particular drug combination, tumors often become simultaneously resistant to many chemically and structurally unrelated anticancer drugs they have never met before. This phenomenon is called *multidrug resistance* (MDR).

The MDR phenomenon is very often caused by the overexpression of certain ATP-dependent drug efflux pumps belonging to the ABC superfamily of transporters. These transporters can bind and extrude various chemotherapeutic compounds directly from the cell membrane before the drugs could reach their intracellular targets. In humans the expression of three membrane transporters including ABCB1 (P-glycoprotein, Pgp), ABCC1 and ABCG2 is responsible for the development of MDR in the majority of cases. The expression level and pattern of the above three transporters in cancer cells often correlates with the tumor grade, and it is a biological marker which can be used for prediction of tumor prognosis.

On the other hand, Pgp, ABCC1 and ABCG2 are also expressed in various normal tissues with barrier function, such as the blood-brain, blood-placenta and blood-testis barriers, choroid plexus, intestinal epithelium, liver and kidneys. The major physiological function of these transporters in accordance with their localization is to provide general protection against xenobiotic compounds. In addition, many drugs that are applied for the treatment of various diseases are also substrates for one or more of the above transporters. Taken together the detailed understanding of the function of these transporters has particular pharmacological importance.

48 ABC protein genes were identified in the human genome and they were classified into seven subfamilies from ABCA to ABCG depending on their sequence similarities. The majority of ABC proteins have been shown to function as ATP-dependent pumps, but an ion channel and channel regulators were also identified among them. The human Pgp is the first discovered human ABC transporter. Because of its medical relevance it is continuously in the focus of intense research. The detailed understanding of structure-function relationships in

Pgp may also shed light on the possible mechanism of polyspecific drug transport that is also typical in certain other members of the ABC superfamily.

1.1 The structure of Pgp

Pgp encoded by the human *MDR1* gene, contains 1280 amino acids and consists of two similar halves separated by a short linker region containing multiple charged residues and phosphorylation sites. Structural analysis of Pgp demonstrated that the protein has two transmembrane domains (TMD) each containing six transmembrane α-helical segments (TM1-TM12), and two cytoplasmic nucleotide-binding domains (NBD) that bind and hydrolyse ATP. The energy released by the binding and hydrolysis of ATP is used to transport the substrates across the plasma membrane. The polyspecific recognition of the transported drugs and Pgp inhibitors occurs in a central cavity formed by the two TMDs, but the precise locations and nature of these binding sites remain poorly defined. Each nucleotide-binding domain contains several conserved motifs including the Walker A, Walker B, signature motif (C motif), A-, Q-, D- and H-loops. These sequences are involved directly or indirectly in ATP binding, in the cross-talk between the NBDs or in the interdomain communication between NBDs and TMDs. The two NBDs form two ATP binding sites cooperatively, since the Walker A motif of one of the NBDs is linked to the signature element of the opposing NBD via a bound ATP molecule.

Determination of the structure of transmembrane proteins is an extremely difficult challenge for researchers. Despite accumulating significant amount of structural data about human Pgp, there are no high resolution structures available that are based on x-ray crystallography data. Therefore, in molecule dynamic simulations homology models based on the crystal structures of bacterial ABC transporters or Pgps from mice or *C. elegans* are used. According to these studies, it is presumable that binding and hydrolysis of ATP at the NBDs can induce conformational changes in the TMDs changing the accessibility of the substrate binding site.

1.2 Catalytic cycle of Pgp and detection of conformational changes by UIC2 antibody

Two stable conformers of Pgp can be assumed in the catalytic cycle of Pgp: the nucleotide-free, inward-facing conformation, and the nucleotide-bound, outward-facing conformation. Based on the results obtained in mouse studies, each of the drug-free and drug-bound state of the protein represents the inward-facing conformational state, while binding of nucleotides to NBDs rearranges TMDs opened toward the EC space which result in the reduction of substrate binding affinity. The catalytic cycle is driven by ATP hydrolysis, which

may happen at either of the two functionally symmetrical composite nucleotide binding sites. However, whether Pgp hydrolyses one or two (or more) ATP molecules per each transported substrate is not known. On the other hand, the NBDs of Pgp molecules crystalized in the absence of substrate and ATP appears to be separated (apo-Pgp). If this conformation is indeed relevant and part of the catalytic cycle, it is possible that both ATPs are hydrolysed during the cycle or alternatively after the hydrolysis of the first ATP the second one is unable to keep the NBD dimer together.

Mutagenesis is an invaluable tool to modify genes and study the structural and functional properties of a protein. To explore the catalytic cycle of Pgp, numerous biochemical experiments were carried out using mutant Pgp variants, in which certain key amino acids of the conserved sequences of one or both NBDs were exchanged. Based on literature, mutation of the highly conserved Walker A lysine residue to methionine in one of the two sites is sufficient to abolish ATPase activity of Pgp and reduce the affinity of ATP binding, assuming that the two NBDs hydrolyse ATP in a strictly alternating manner.

UIC2, a conformation sensitive anti-Pgp mAb, when applied at saturation binds only to 10-40 % of Pgps present in the cell membrane of intact cells. Rest of the Pgp molecules become recognized by this antibody in ATP-depleted cells or when cell are labeled by UIC2 in the presence of certain substrates or modulators (including vinblastine, CsA). Simultaneous treatment of cells with Pgp modulators added at sub-inhibitory concentration increases the UIC2 reactivity of cells and inhibits Pgp mediated substrate transport. Previous studies showed that the UIC2 reactivity decreases in the presence of increasing concentration of ADP, ATP or non-hydrolysable ATP analogues (AMP-PMP) in permeabilized Pgp expressing cells. All of these data suggest that catalytic cycle of Pgp presumably involves conformational changes which characterized by different UIC2 affinity.

1.3 Localization of Pgp in rafts and non-raft membrane microdomains

In view of the intimate association of Pgp with the lipid bilayer in which it is embedded, and from which it harvests its substrates, the function of Pgp is determined by the characteristics of the membrane. Accordingly, the membrane microenvironment affects the conformational changes and transport activity of Pgp, the distribution of Pgp substrates in the lipid bilayer and their interaction with the protein. Furthermore, the binding and hydrolysis of ATP can also be influenced by the interaction of the membrane with NBDs or by the stabilization of transmembrane alpha-helices. Substrate stimulation or inhibition of the

ATPase activity of Pgp also can be influenced by the lipid environment. The presence of cholesterol in the membrane decreases the membrane permeability by filling the gaps between phospholipids molecules, therefore decreases the partitioning of certain Pgp substrates (e.g. CsA) into the lipid bilayer. In contrast, removal of cholesterol from the membrane by methyl-beta-cyclodextrin increases vincristine uptake into the control cells and the Pgp expressing cells alike. In conclusion, since membrane properties have a complex effect on Pgp function, changing the properties of the cell membrane may be a good approach to reduce or overcome multidrug resistance in clinical practice.

Certain lateral membrane microdomains, such as the „lipid rafts" and caveolae differ from the neighbouring membrane regions due to their high content of GPI-anchored proteins, cholesterol and sphingolipids. „Lipid rafts" and caveolae are involved in signal transduction, transport and membrane trafficking processes. „Lipid rafts" are composed of a highly ordered, Triton X-100-insoluble core region, which is surrounded by a less structured shell that is resistant to milder detergents (e.g. Brij 98). Several previous studies suggest that the majority of Pgps reside in the Brij96-resistant membrane microdomains and the rest of Pgps found in the less ordered shell area, or completely outside of rafts. The location of Pgps in different membrane microdomains probably suggests their heterogeneous function, while others suppose that only the raft-associated Pgp molecules are active. This latter hypothesis raises the possibility that altering the distribution of Pgps between raft and non-raft membrane microdomains can be a good approach to modify Pgp function.

2. Objectives

Although, the catalytic cycle of Pgp is widely studied, the molecular mechanisms linking ATP binding and/or hydrolysis to the association and dissociation of NBDs and to conformational changes occurring in the TMDs are not fully understood. How ATP hydrolysis is coordinated between the two NBDs, and whether Pgp hydrolyses one or two (or more) ATP molecules per each transported substrate are still not known.

In the first part of our work we studied the conformational changes of Pgp during its catalytic cycle by the UIC2 conformational sensitive monoclonal antibody in cells permeabilized by *Staphylococcus aureus* alpha toxin to answer the following questions:

1. Which catalytic event can drive Pgp molecules from the inward (UIC2-binding) to the outward facing (UIC2-dim) conformation?
2. Whether the binding of monoclonal antibodies (15D3, 4E3) recognizing overlapping epitopes with UIC2 can be influenced by the ATP-dependent conformational changes of the transporter?
3. How do the single and double Walker A mutations affect the transition of Pgps from the UIC2 binding conformation to the UIC2-nonbinding conformation?
4. Whether the kinetics of formation or dissociation of the post-hydrolytic complexes trapped by phosphate mimicking anions differs in wild-type and Walker A mutant Pgps?

In the second part of this work our aim was to characterize the UIC2-bound and UIC2-dim Pgp pools present in the membrane of intact cells answering the following questions:

5. Is there a correlation between the intracellular ATP concentration and the UIC2 reactivity of Pgps in intact cells?
6. Whether the modification of the intracellular ATP concentration affects the association of Pgp molecules to the detergent-resistant raft membrane microdomains?
7. Whether mutation of the Walker A lysine in both NBDs affects the association of Pgp molecules to the different membrane microdomains?
8. Whether the UIC2 reactive and UIC2-dim Pgp pools differ in their internalization rate?

3. Materials and methods

3.1 Cell lines

The *MDR1* gene-transduced NIH 3T3 mouse fibroblast cell line (NIH 3T3 MDR1 G418, a gift from M. Gottesman), the KB-3-1 human epidermoid carcinoma cell line and its vinblastine resistant subline (KB-V1) were used in these experiments. Cell lines stably expressing the wild-type, K433M, K1076M, and K433M/K1076M double mutant Pgp variants were established by the Sleeping Beauty (SB) transposon-based gene delivery system, using the 100 fold hyperactive SB transposase.

The cells were grown as monolayer cultures at 37°C in an incubator containing 5% CO_2, and were maintained by regular passages in Dulbecco's modified Eagle's medium (DMEM) supplemented with heat-inactivated fetal calf serum, L-glutamine, and penicillin-streptomycin cocktail. To maintain the high expression level of Pgp molecules NIH 3T3 MDR1 cells were cultured in a medium containing 670 nM doxorubicine, while KB-V1 cells were maintained in the presence of 180 nM vinblastine. Two or three days before the experiments medium was replaced by drug-free culture medium. Cell lines were grown as monolayer cultures and were harvested with trypsin-EDTA before use. The FRET-based ATP sensor was expressed in KB-V1 cells using a lentiviral expression system.

3.2 Chemicals

All chemicals, cell culture media and supplements were from Sigma-Aldrich (Budapest, Hungary). The UIC2, 4E3 and 15D3 anti-Pgp mAbs were prepared from the supernatants of the hybridomas (obtained from the American Type Culture Collections (Manassas, VA, USA)) and were >97% pure by SDS/PAGE. The antibodies were labeled with Alexa 488 or Alexa 647 succinimidyl ester (A488 or A647, Life Technologies, Inc. (Carlsbad, CA, USA)) and separated from the unconjugated dye by gel filtration on a Sephadex G-50 column. The dye-to-protein labeling ratio was around 3 for each antibody preparation. Unless otherwise stated antibodies were used at quasi saturating concentrations: 10 µg/ml UIC2, 60 µg/ml 4E3 and 15 µg/ml 15D3). RAMEB (Methyl-beta-cyclodextrin) and its cholesterol containing complex (Cholesterol-RAMEB: cholesterol content 5.10 %, determined by HPLC) were from Cyclolab Ltd. (Budapest, Hungary). Non-ionic detergents were purchased from Pierce Biotechnology, Inc. (Kvalitex Ltd, Budapest, Hungary).

3.3 Permeabilization of cells with *Staphylococcus aureus* α-toxin

Cell suspensions were treated with α-toxin in phosphate-buffered saline (PBS) in the presence of 1 % bovine serum albumin (BSA) at 37 °C for 30 min, allowing permeabilization of approximately 50% of the cells (judged by propidium iodide (PI) positivity). The reaction was stopped with PBS and the cells were centrifuged (at 2250×g) at room temperature. Unbound toxin was removed by washing the cells with PBS and the cell pellet was re-suspended in PBS.

3.4 Determination of the apparent affinity of nucleotide binding

Permeabilized cells were pre-incubated for 20 min with nucleotides added in a broad concentration range and then further incubated at 37 °C for 30 min with A647-conjugated UIC2 monoclonal antibody. To prevent ATP hydrolysis, ATP was added to permeabilized cells without Mg^{2+} in the presence of 5 mM EDTA. Alternatively, ATP was replaced by the non-hydrolysable ATP analogue AMP-PNP/Mg^{2+}, or the experiment was conducted on ice. In the latter case the labeling of cells with A647-conjugated UIC2 monoclonal antibody was carried out at 4 °C for 45min. Following antibody labeling, samples were washed 3 times with ice-cold PBS. The UIC2-A647 fluorescence intensity of PI-positive permeabilized cells was measured by flow cytometry and plotted as a function of the nucleotide concentration. To determine the apparent affinity of Pgp to the nucleotides data points were fitted with a four-parameter Hill function.

3.5 Nucleotide trapping

The post hydrolytic transition state of Pgp was stabilized by phosphate analogues, vanadate or BeF_x. Permeabilized cells were incubated with ATP/Mg^{2+} and V_i or BeF_x the presence or absence of substrates in PBS at 37°C. Aliquots were taken at regular intervals and washed twice with ice-cold PBS containing V_i or BeF_x at the same concentrations that were applied previously during incubation. Samples were re-suspended in ice-cold PBS and labeled with UIC2-A647 monoclonal antibody in the presence of the previously applied phosphate analogue at 4 °C for 45min. The UIC2-A647 fluorescence intensity of the samples was measured by flow cytometry and plotted as a function of time.

3.6 Recovery from the V_i -trapped post-hydrolysis state

Permeabilized cells trapped in the presence of ATP/Mg^{2+} and V_i were washed 3 times with ice-cold PBS and incubated in the presence or absence of ATP and/or substrates (verapamil, vinblastine) at 37 °C. Aliquots were taken at regular intervals and washed twice

with PBS. The aliquots were incubated in the presence of 200 μM N-ethyl-maleimide to avoid *de novo* disulphide bond formation between Walker A cysteines and labeled with UIC2-A647 at 37 °C for 15 min or at 4 °C for 45min. At the end of incubations the samples were washed three times with ice-cold PBS and kept on ice until measurement in the flow cytometer.

3.7 Detergent elution procedure (FCDR, Flow cytometric detergent resistance assay)

After labeling with anti-Pgp antibodies (UIC2-A647, 4E3-A647 or 15D3-A647) samples were washed twice with PBS and divided into two aliquots. One aliquot was re-suspended in ice cold PBS, while the other was treated with 0.5% TX-100 in PBS at 4 °C for 20 min. Subsequently the samples were fixed with freshly prepared 4 % paraformaldehyde solution. Fluorescence intensity of cells was measured in a flow cytometer. The detergent resistant fraction of Pgps was calculated as a ratio of the mean fluorescence intensities of the TX-100 treated and untreated cells.

3.8 Effect of modification of intracellular ATP concentration on cell surface Pgp molecules and association of TX-100 resistance membrane microdomains

Cells were treated with 10 mM 2-deoxy-D-glucose for different time and washed them twice with PBS. After washing procedure cells were labeled with 4E3-A647 antibody at 37 °C for 30min. 4E3-A647 fluorescence intensity was plotted as a function of starvation time.

3.9 Relationship between UIC2-reactivity of Pgp molecules and intracellular ATP concentration

We used a FRET-based ATP sensor to detect the changes of the intracellular ATP concentration in intact KB-V1 cells. In order to decrease intracellular ATP concentration cells were treated with 2-deoxy-D-glucose alone or in combination with potassium cyanide in PBS. Control samples were prepared in the presence of 0 mM or 8 mM glucose without 2-deoxy-D-glucose. All of the samples were treated with 100 μg/ml UIC2-A647 antibody at 37°C, for 3 min. After labeling cells were washed and fluorescence intensity of ATP sensor and binding of antibody was detected by flow cytometry.

3.10 Internalization of Pgp molecules

The percentage of endocytosed Pgps was determined after labeling the cells with Alexa-647-conjugated UIC2 or 15D3 antibodies. After modulation of the cellular cholesterol levels by treatment with 5 mM RAMEB and Chol-RAMEB (Cyclolab Ltd. (Budapest, Hungary)), the antibodies bound on the cell surface were removed by washing the cells with a low pH buffer (0.5 M NaCl, 0.1 M glycine, pH=2.5). The mean fluorescence intensities of the

low pH buffer treated and untreated samples were determined in a flow cytometer and the fraction of fluorescence intensity with standing the acidic treatment was calculated to yield the percentage of internalized receptor-antibody complexes.

3.11 Flow cytometry

Fluorescence intensity of cells was measured by using a Becton Dickenson FACS Array flow cytometer; the data were analysed with the BDIS CellQuest software. A 635nm laser was used for the excitation of the Alexa 647 dye and the fluorescence was detected in the red channel (661/16 nm), while the 532 nm laser was used for the excitation of PI (detected at 585/42nm). Cell debris was excluded from analysis on the basis of FSC and SSC signals. The median fluorescence indicating UIC2 reactivity was determined in PI positive cells. In the case of permeabilized cells propidium-iodide positive cell population was analysed, while in the case of intact cells propidium iodide negative cell population was analysed. Becton Dickinson FACS Aria flow cytometer was used to detect the fluorescence intensity of ATP sensor and antibody binding simultaneously. A 445nm laser was used for the excitation of YFP and the fluorescence was detected in the red channel (530/30 nm), while the 631 nm laser was used for the excitation of Alexa 647 dye and the fluorescence was detected in the red channel (660/20 nm).

3.12 Data and statistical analysis

Data were analysed using SigmaStat (version 3.1, SPSS Inc., Chicago, IL, USA) and are presented as means± SD. Comparison of two groups was carried out by unpaired t-test, statistical significance in the case of three or more groups was assessed using analysis of variance (ANOVA), applying the Holm-Sidak multiple comparison test for post hoc pair-wise comparison of the data. Differences were considered significant at $P< 0.05$.

4. Results I

4.1 UIC2-detectable conformational transitions in TMDs reflect processes in NBDs

Gentle permeabilization of cells by *Staphylococcus aureus* alpha-toxin allows selective modulation of the intracellular milieu while preserving membrane integrity and the function of transmembrane transporters. Permeabilized cells lose their ATP content, the NBDs of Pgp become nucleotide-free and these Pgps are synchronized in an ATP-free, high UIC2 affinity conformation. We applied ATP/Mg^{2+} in a broad concentration range and measured the binding of the fluorescent dye conjugated UIC2 mAb to the permeabilized cells, which is decreased in a concentration dependent manner. The apparent affinity of ATP for competing UIC2 labeling was found to be comparable with reported K_M value for ATP-hydrolysis obtained in membrane samples prepared from Pgp^+ NIH 3T3 cells. We couldn't detect similar effect when we used 15D3 or 4E3 antibody (control antibody), which can recognize overlapping epitopes with UIC2 on the Pgp molecule. In the further experiments we applied 15D3 and 4E3 as control antibodies to verify the actual occurrence of the conformational changes detected by UIC2.

4.2 Control experiments

In our control experiments we couldn't detect decrease in UIC2 reactivity in the presence of increasing concentration of adenosine-monophosphate, probably because adenosine-monophosphate is not able to induce the dimerization of NBDs and the concomitant UIC2 detectable conformational changes of TMDs. However, the binding of adenosine-diphosphate was sufficient to induce the switching of Pgp molecules to the UIC2-dim conformation.

N-ethyl-maleimide (NEM) is a sulfhydryl reagent that binds covalently to the cysteine residue of the Walker A motifs when the nucleotide binding site is empty or binds ADP. In the presence of NEM UIC2 reactivity increases in a concentration dependent manner. Since the Walker A motif interacts with the beta and gamma phosphate of ATP pre-treatment of permeabilized cells with ATP prevents the effect of NEM on UIC2 reactivity, which manifested in a significant right shift of the UIC2 binding curves. All of these data suggest that our assay is sensitive enough to detect the TMD conformation changes of Pgp brought about by manipulation of the nucleotide binding site.

4.3 Nucleotide binding switches Pgp into the outward facing conformation

AMP-PNP, a non-hydrolysable ATP analogue induced a conformation change in the transmembrane domains resulting in a concentration dependent decrease in UIC2 reactivity. When ATP was added in the absence of Mg^{2+} or on ice, i.e., under conditions that prevent ATP hydrolysis, the conformational change also driving Pgp into a UIC2-reactive state was observed at significantly higher nucleotide concentrations. The transport and ATPase cycle of Pgp is inhibited by phosphate-mimicking anions, such as orthovanadate or BeF_x, which can block the protein by stably replacing the cleaved gamma phosphate. The complex consisting of Pgp, ADP and vanadate is formed only under conditions that allow hydrolysis of at least one ATP molecule. Consistent with the formation of the Pgp-ADP-V_i complex, addition of vanadate increased the apparent nucleotide affinity under hydrolysis conditions. However, when ATP hydrolysis was prevented, the apparent nucleotide affinity was not influenced at all by V_i, confirming that ATP hydrolysis and the subsequent release of the γ-phosphate is a prerequisite for the formation of the Pgp-ADP-V_i complex.

4.4 Unilateral mutation of the Walker A lysine residues allows ATP binding and substrate-stimulated ATP hydrolysis.

The Walker A motif forms extensive bonds with the terminal phosphates of the bound nucleotide. Mutation of the highly conserved lysine residue to methionine in the Walker A motif was shown to abolish ATPase activity of several ABC transporters. To study the effect of mutations of the Walker A lysines in NBD1 (K433) and NBD2 (K1076) of Pgp on nucleotide-induced TMD conformational flexibility, we applied mammalian cell lines (NIH 3T3) stably expressing wild-type, K433M, K1076M, or K433M/K1076M P-glycoprotein using a *Sleeping Beauty* transposon-based gene delivery system. Consistent with previous studies that showed that single Walker A mutations allow nucleotide binding, UIC2-reactivity of the K433M and K1076M variants decreased in the presence of AMP-PNP and ATP. Furthermore, we have found that addition of vanadate resulted in a 5–10-fold increase in the apparent affinity of the single mutants to ATP. Because complex formation with vanadate or BeF_x requires prior hydrolysis of ATP, these effects of vanadate indicate that Pgp is capable of ATP hydrolysis despite the mutation of a single Walker A lysine residue. In contrast, simultaneous mutation of both lysine residues resulted in a stable UIC2-binding conformation, which could not be reverted by nucleotides.

4.5 Single Walker A mutants are transport-competent.

The turnover rate of Pgp's catalytic cycle is accelerated in the presence of transported substrates (substrate-stimulated ATPase activity). Kinetic analysis of the formation of the BeF_x- or V_i-trapped complexes showed that progressive accumulation in the low-UIC2-affinity trapped transition state occurs at comparable rates in WT or single mutant Pgps. Accumulation of the trapped complex was accelerated by Pgp substrates (e.g. verapamil or vinblastine) both in WT and single Walker A mutant Pgp variants, while the apparent affinity for ATP did not change even at high substrate concentrations. These data suggest that despite the mutation of a single critical Walker A lysine residue, the catalytic cycle can progress to the step arrested by phosphate mimicking anions. The relatively long $t_{1/2}$ values of BeF_x and V_i trapping compared to the total cycle time suggest that formation of a stable post-hydrolysis complex by phosphate mimicking anions is an extremely low-probability event, likely reflecting the very short time window of the vanadate- or BeF_x-"sensitive" state in each cycle, that is between dissociation of the cleaved phosphate and disassembly of the NBD dimer. Thus, similarly to WT Pgp, single Walker A mutant Pgp variants can not only hydrolyse ATP, but pass on average ~1200–2500 cycles before trapping by BeF_x or vanadate occurs. Consistently with this turnover, single mutants retain a weak, but significant verapamil stimulated ATPase activity that can be detected in membrane samples prepared from NIH 3T3 cells expressing the transporter at high levels.

4.6 The rate of dissociation of the V_i -trapped complex in wild-type and single mutant Pgps

Although the V_i -trapped species represent a dead-end of the catalytic cycle, we wondered if the eventual release of the trapped nucleotide could be detected by an increase of UIC2 reactivity. Following the removal of uncomplexed V_i, complete time-dependent recovery of the UIC2-reactive state corresponding to the release of nucleotides was indeed observed. UIC2 binding to the mutant Pgp variants was not restored in the time frame of the experiments, suggesting that mutation of a single Walker A residue increases the life time of the vanadate-trapped complex.

4. Results II

4.7 Distribution of double Walker A mutant Pgps between membrane microdomains with different detergent solubility

UIC2 mAb binds only to 10–40% of cell surface Pgp molecules („pool-I"), while in the presence of Pgp inhibitors like cyclosporine A (CSA) or ATP depleting agents the rest of the cell surface Pgps also become available to UIC2 mAb („pool-II") in various cell lines. The catalytically inactive double Walker A mutant Pgp variants are stabilized in a high UIC2 affinity inward-open conformation regardless of treatments. Previous data of our workgroup suggest that in various cell line (including NIH 3T3 MDR1) 20-50 % of Pgp molecules showed resistance to TX-100 detergent solubilisation, consequently found in the TX-100 resistance rafts/caveolae.

In our experiments 50-60% of „pool-I" Pgp molecules showed resistance to TX-100 detergent solubilisation in Flow Cytometric Detergent Resistance (FCDR) assay. When all Pgps expressed on the cell surface were labeled with 15D3-A647, 4E3-A647 or UIC2-A647 in the presence of CSA only 25-30% of them showed resistance to TX-100 solubilisation. Furthermore, the detergent resistant fraction of Pgps did not depend on the applied antibody. These results suggest that wild-type „pool-I" Pgps are preferentially associated with the TX-100 resistant rafts and the cytoskeleton, as compared to the „pool-II" Pgp molecules. Interestingly, double Walker A mutant Pgps show similar detergent resistance as wild-type Pgp molecules. If the UIC2-reactive Pgp conformer favours the raft milieu, we would expect greater detergent resistant fraction in case of double Walker A mutant Pgps compared to wild-type Pgps, but it was not proved by the experiments.

4.8 Correlation between intracellular ATP content and UIC2 binding in intact cells

In our experiments the non-metabolizable glucose analogue 2-deoxy-D-glucose and glucose were used to modulate intracellular ATP level in intact cells. Changes of the intracellular ATP concentration were detected by a FRET-based ATP sensor, while proportion of UIC2-reactive and non-reactive Pgps molecules were also detected. In our experiments 2-deoxy-D-glucose decreased the intracellular ATP concentration and thereby increased the fraction of the UIC2-reactive Pgps depending on the length of the incubation time. Based on these results, in intact cells there is a strong correlation between the intracellular ATP level and UIC2 reactivity similarly to the permeabilized cells.

4.9 The effect of intracellular ATP concentration on the association of Pgp molecules to the detergent-resistant membrane microdomains

In these experiments we used 4E3 non-conformation sensitive monoclonal anti-Pgp antibody which can bind to all of Pgps expressed on the cell surface. Our results showed that short-term ATP depletion of cells did not affect the cell surface expression level of Pgps and their association to the detergent-resistant membrane microdomains. These results suggest that switch of Pgps into the UIC2-reactive outward-facing conformation doesn't affect the appearance of these Pgps in raft domains; rather the lipid raft environment itself stabilizes Pgp molecules in an ATP-free UIC2-reactive conformation.

4.10 Cholesterol-dependent internalization of the two different Pgp pools distinguished by UIC2-reactivity

To study the internalization of the two Pgp population we used UIC2-A647 antibody to label ,,pool-I" Pgp molecules. Prior to addition of concomitant use of CsA and UIC2 antibody ,,pool-II" Pgps are labeled with UIC2 antibody. All of the Pgps expressed on cell surface are labeled with 15D3-A647 or concomitant use of CsA and UIC2-A647. After labeling, one of the sample pairs was washed with low pH solution to remove antibody from cell surface Pgp molecules. Internalized Pgp fraction was measured by flow cytometry. Based on our results, significantly higher proportion of ,,pool-I" Pgps was internalized compared to ,,pool-II" Pgps labeled by the same antibody, supporting that the two Pgp pools are probably localized in different membrane microdomains. Our study also demonstrated that internalization of Pgp molecules was significantly enhanced by increasing the cholesterol content of the plasma membrane. On the other hand, internalization of ,,pool-I" Pgps was decreased by extraction of membrane cholesterol level, further supporting that internalization of Pgps is a raft/caveolae-dependent process. Pgp molecules show higher internalization when labeling is carried out with UIC2 antibody, suggesting that binding of UIC2 antibody to Pgps stimulates their endocytosis. Interestingly, internalization of Pgps labeled with 15D3 was increased more significantly by loading of the cell membrane with cholesterol.

5. Discussion I

Based on structural studies and a wealth of biochemical and biophysical data it is generally accepted that ABC transporters have at least two discrete conformations: inward-facing conformation characterized with dissociated NBDs opened toward the cytoplasm and outward-facing conformation characterized with dimerized NBDs and rearranged TMDs opened toward the extracellular space. The switch between the inward- and outward-facing conformations involves a series of conformational changes which ultimately result in reduction of the substrate binding affinity required for uphill substrate transport. However, the exact molecular mechanisms that link nucleotide binding to the association and dissociation of NBDs and to the conformational changes of the TMDs are not fully understood.

In the present study we used the UIC2 conformation-sensitive monoclonal antibody to detect molecular movements of the TMDs which reflects conformational changes of the nucleotide binding sites. UIC2 distinguishes two conformations of Pgp, which can be studied by modulating intracellular ATP-levels in *Staphylococcus aureus* alpha-toxin-permeabilized cells overexpressing Pgp. In our experiments the catalytic cycle of Pgp was shalted at different stages by using non-hydrolysable ATP analogues, phosphate mimicking anions or mutant Pgp variants and the conformation of these intermediates was analyzed by UIC2 reactivity. The substrate binding affinity of the different catalytic intermediates in wild-type and mutant Pgp variants was studied by my co-workers using fluorescent Pgp substrates and confocal microscopy.

Our results demonstrate that AMP-PNP binding to Pgp is sufficient to induce the conformational switch from the inward to the outward facing conformation. Similar results were obtained when ATP hydrolysis was prevented by using ATP without magnesium or ATP/Mg^{2+} at 4°C. Using the same experimental setup my colleagues observed that binding of AMP-PNP switched Pgp into a low drug binding affinity state. The simultaneous drop in the UIC2-reactivity and drug-binding affinity suggests that the transition from the inward- to the outward-facing conformation precedes ATP hydrolysis in any case.

Residues of the Walker A motifs in each NBD, together with the signature sequence of the contralateral NBD, directly participate in nucleotide-dependent dimerization of the two NBDs and ATP hydrolysis. How ATP hydrolysis is coordinated between the two NBDs, and whether Pgp hydrolyses one or two (or more) ATP molecules per each transported substrate are not known. The two nucleotide binding domains of Pgp were shown to be functionally

equivalent and the integrity of both catalytic centers is generally believed to be needed for transport, because inactivation of a single NBD results in inhibition of ATPase and transport activities.

We confirmed that mutation of both Walker A lysine residues inactivates Pgp, the transporter is essentially frozen in the UIC2-reactive inward-open state characterized with high drug binding affinity. However, our data show that nucleotide binding to single Walker A mutants triggers the same inward-to-outward conformational switch, and the concomitant drop in drug binding affinity, as observed for the wild-type protein. Furthermore, we demonstrated that vanadate exerts the same effect on the UIC2 binding curves of the wild-type and the unilateral Walker A mutant Pgp variants. Since vanadate-dependent trapping is an extremely low-probability event, formation of the stable V_i -trapped complex, as well as the significant left shift of the UIC2 binding curves in the presence of V_i, can only be explained if single Walker A mutants are catalytically competent. Admittedly, this result is opposed to prevailing views insisting on the requirement of two intact nucleotide binding domains for ATP hydrolysis by Pgp. However, we note that the majority of the studies reporting the inactivating effect of single Walker A mutations have been performed using heterologous expression systems such as Sf9, *Saccharomyces cerevisiae* or purified and reconstituted proteins. It is known that the plasma membrane composition influences the catalytic activity of ABC transporters, and that membrane cholesterol amounts influence Pgp activity. Since membrane cholesterol levels are significantly lower in lower eukaryotes, it may be that the low ATPase activity of the single Walker mutants was missed due to the different plasma membrane composition of the heterologous expression systems, or artefacts related to the solubilization, purification and reconstitution of the proteins.

Vanadate-trapped species are formed with comparable rates in wild-type and single mutant, suggesting that the fraction of time the working transporter spends in the V_i -sensitive post-hydrolytic state is similar for wild-type and single mutant Pgp. Interestingly however, the rate of disassembly of the V_i -trapped complex is greatly reduced in the single mutants. Assuming that the trapped complex harbors an ATP in the non-committed site, one might speculate that disassembly of the complex happens only upon eventual hydrolysis of that ATP. This process might happen at a rate of ~1 per hour in a non-committed wild-type catalytic center, but never at all in a center disabled by a Walker A mutation. Taken together our experiments carried out with single Walker A mutants suggest that while one hydrolysis event is sufficient to reset the high-substrate affinity inward facing conformation in the

absence of V_i , dissociation of the V_i -trapped complex also requires the hydrolytic activity of the contralateral site, supporting the possibility that the V_i -trapped complex may not mimic a true catalytic transition state in case of Pgp. However, much further work will be required to fully understand these details.

Our results are incompatible with catalytic cycle models supposing the hydrolysis of two ATPs per cycle. Our results are also difficult to reconcile with models suggesting that the two NBDs hydrolyze ATP in a strictly alternating order. If the NBDs were indeed recruited in a strictly alternating fashion, every second ATP would have to be processed by the mutant catalytic center, causing the cycle to stall. Instead, our results indicate that the wild-type catalytic site can hydrolyze ATP in repeated cycles without hydrolysis at the other NBD. The simplest interpretation of our data is that in WT Pgp one of the two functionally equivalent sites becomes committed to hydrolysis in each cycle on a random basis, whereas in the single mutants commitment of the only functional site initiates every cycle.

5. Discussion II

In cell membrane of various cell lines Pgp molecules behave as a “cosmopolitan” and present in the TX-100 resistant, presumably raft- and cytoskeleton-associated membrane microdomains and in the TX-100 soluble membrane microdomains as well. In the second part of our study we have investigated the relationship between the conformation, the membrane microdomain localization and the endocytosis of Pgp.

In previous studies our workgroup has demonstrated a preferential association of ATP-free UIC2-reactive Pgps with lipid rafts. The rest of Pgp molecules („pool–II” Pgps) become UIC2-reactive only in the presence of certain inhibitors such as CsA or ATP-depleting agents and are localized mostly in the non-raft membrane microdomains („pool–II” Pgps). These data rise the question whether the UIC2-reactive, catalytically dead double Walker A mutant Pgps show higher raft- and cytoskeleton-association compared to the wild-type transporter. Our results showed that catalitically inactive double Walker A mutant Pgps exhibited similar detergent resistance compared to the wild-type transporter.

Our studies also demonstrated that treatments with 2-deoxy-D-glucose decreased the intracellular ATP concentration in a time dependent manner and thereby increased the fraction of the UIC2-reactive Pgps. However, short term starvation of cells did not affect the cell surface expression of Pgps and their association to the detergent-resistant membrane microdomains. These results suggest that switch of Pgps into the UIC2-reactive outward-facing conformation does not drive them to the raft microdomains, rather the lipid raft milieu itself can stabilize Pgp molecules in an ATP-free UIC2-reactive conformation.

Furthermore, we have demonstrated that internalization of Pgps labeled by UIC2 antibody is 4-6 times faster compared to the 15D3 antibody labeled Pgps, suggesting that binding of UIC2 antibody promotes their internalization. Endocytosis of raft-associated “pool-I” Pgps was the highest, therefore endocytosis of Pgps probably facilitated by the raft milieu. Consistent with our hypothesis, we also demonstrated that increasing the cholesterol concentreation of the plasma membrane facilitates the internalization of Pgps. This effect was the most significant in the case of “pool-II” Pgps and when all of Pgps in the cell membrane were labeled by 15D3 antibodies. Internalization of Pgps was decreased by extraction of membrane cholesterol by RAMEB and this effect was the most pronounced in the case of “pool-I” Pgps. Taken together these data, it seems likely that the internalization of Pgp molecules probably happens with participation of cholesterol-rich rafts and caveolae.

6. Summary

We have applied the UIC2 conformation selective monoclonal antibody to elucidate the conformational changes of Pgp at different steps of its catalytic cycle using permeabilized cells. We have made the following statements regarding the catalytic mechanism of Pgp:

1) The conformational rearrangements of Pgp responsible for switching between inward facing, high substrate affinity state and the outward facing low substrate affinity conformation occur prior to the ATP hydrolysis event.
2) The binding of the 4E3 and 15D3 monoclonal antibodies recognizing extracellular epitopes partially overlapping with the epitope of UIC2 is not sensitive to the ATP-dependent conformation changes of Pgp.
3) Similarly to wild-type Pgp, nucleotide binding drives the single Walker A mutant Pgp variants from the inward to outward-facing conformation. In contrast, simultaneous mutation of both lysine residues stabilizes the transporter in a high substrate affinity inward-open conformation.
4) Addition of phosphate mimicking anions such as V_i or BeF_x results in a 5–10-fold increase in the apparent nucleotide affinity indicating the formation of a stable posthydrolytic complex either in wild-type or the the single Walker A mutant Pgp variants.
5) The rate of disassembly of the V_i-trapped complex is greatly reduced in the single mutants compared to the wild-type Pgp supporting the possibility that resetting the inward facing conformation requires two intact catalytic sites.

In the second part of our work, we have investigated the relationship between the conformation, the membrane microdomain localization and the endocytosis of Pgp.

6) In intact cells similarly to permeabilized cells, there is a close correlation between the intracellular ATP concentration and the UIC2 reactivity of Pgp molecules.
7) Short-term ATP depletion of cells that switches Pgps into a UIC2-reactive conformation does not affect the cell surface expression of Pgp molecules and their association to the TX-100 resistant membrane microdomains.
8) Double Walker A mutant and wild type Pgps show similar detergent resistance (approx. 25-30%) supporting their similar membrane microdomain localization.
9) ”Pool-I” Pgp molecules exhibit faster endocytosis compared to „pool-II” Pgps probably indicating their distinct membrane microenvironment.

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   Sci. Rep. 6 (24810), 1-16, 2016.
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   Biochim. Biophys. Acta. Mol. Cell Biol. Lipids. 1861 (3), 188-195, 2016.
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