EFFECTS OF THE Q141K AMINO ACID CHANGE IN THE ABCG2 TRANSPORTER

Doctoral thesis
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Introduction

ABC proteins are integral membrane proteins and transport compounds across the membrane utilizing the energy of ATP binding and hydrolysis. ABC proteins form one of the largest protein families, represented in all living organisms from bacteria to human [1]. Prokaryotes take up the essential nutrients (for example sugars and amino acids) from the environment employing ABC importers and they eliminate the toxic molecules by their ABC exporters. In eukaryotes, ABC proteins localize in the plasma membrane and some of them are also found in the membranes of various cell organelles. They facilitate the relocation of molecules across membranes which processes include extrusion of metabolic products and xenobiotics from the cell. Most of the human ABC proteins work as exporter membrane proteins, but ion channels, receptors and cytoplasmic translation factors are also present in this family. The Human Genome Organization categorizes the ABC proteins into seven subfamilies (A-G) based on their sequence similarity [2].

One of the main human multidrug transporters is the ABCG2, which has an important role in protecting the cell and various tissues by eliminating toxic agents. ABCG2 transports various drugs altering their absorption, distribution and excretion. Furthermore, among its substrates, there are antiproliferative chemotherapeutic agents such as mitoxantrone, anthracyclines (doxorubicin, daunorubicin), tyrosine-kinase inhibitors (imatinib, nilotinib, gefinitib), flavonoids (quercetin, genistein), α-blockers (prazosin) and various fluorescent substances (Rhodamin 123, Hoechst 33342) [3]. However, it also takes part in the transport of endobiotics. As one of the urate transporters, ABCG2 participates in the urate excretion in the kidney and the colon [4, 5], and there are porphyrin derivatives (hem group from hemoglobin [6] and pheophorbide-a from chlorophyll [7]) among its substrates. Moreover, there is evidence of transport of riboflavin [8] and estrogen [9] by ABCG2.

The presence of ABCG2 has been shown in mammary glands, in ovary, testis, kidney, liver, small intestine, colon, stem cells and the blood-brain barrier, blood-testis barrier and placenta, where it has important role in protecting these organs and the embryo [10-13].

ABCG2 is localized in the plasma membrane. This transporter is a half transporter, the monomer form possesses a transmembrane domain (TMD) and an intracellular nucleotide binding domain (NBD), and it consists of 655 amino acids (Mw: 72 kDa). The NBD is localized at the N-terminus (amino acids: ~ 1-299) followed by a linker region before the
TMD, which is positioned C-terminally [14]. Two polypeptide chain with one TMD and one NBD form a functional homodimer [15-17].

The TMDs bind and translocate the substrate, providing the translocation pathway through the membrane bilayer. The process is powered by ATP bound in the NBDs. Three conservative sequences take important roles in the catalytic cycle: Signature, Walker A and Walker B sequences. In the functional dimer, two NBDs form two ATP binding pockets which in two ATP molecules are each sandwiched between the Walker A motif in one of the NBDs and the Signature motif in the other NBD. The most important amino acids in the Signature motif \((^{186}VSGGE^{190})\) are the S187, G188 and G189 which attack the \(-\)phosphate of ATP bound in the Walker A \((^{80}GPTGGGKS^{87})\) on the other NBD, resulting ATP hydrolysis [18].

The ATP binding and hydrolysis induce conformational changes in the NBDs which propagates to the TMDs altering the arrangement of the transmembrane helixes. As a result, the substrate is translocated to the extracellular space. ATP binding and hydrolysis are continuously ongoing in the absence of substrate at a low level and the presence of a substrate increases this basal ATPase activity. The ATPase cycle and the transport function of ABCG2 can be inhibited by specific inhibitors, such as Ko143 [19].

Studying the ABCG2 structure is highly limited similar to other membrane proteins. All the methods for structural investigations (e.g. NMR, X-ray crystallography) are limited by the large size and the hydrophobic nature of these proteins. However, recent developments in single molecule microscopy certainly will deliver breakthroughs in the near future.

There are several known malfunctioning variants of ABCG2. In my studies I focused on the Q141K variant. The glutamine/lysine substitution at the 141 position of the NBD decreases the urate export efficiency thereby increasing the risk of developing gout [4, 20]. In addition, the expression of the Q141K variant results in an increased accumulation of xenobiotics (including therapeutic agents) in cells and also in specific tissues [21, 22]. Two main reasons have been determined in the background of the decreased substrate transport. The Q141K variant is presented at a lower level in the plasma membrane and its ATPase
activity is reduced as compared to the wild type [21, 23-27]. It has been demonstrated that the mRNS levels of the WT and Q141K are similar [28], thus the negative effects derive from alterations in the protein conformation.

The Q141 is localized next to a conserved phenylalanine (F142), which is homologous to the well-characterized F508 in the CFTR (ABCC7) ion channel. The deletion of this F508, which causes cystic fibrosis, disrupts the NMD/TMD interface destabilizing the conformation of the protein. As a result, the unstable and dysfunctional ΔF508 protein is degraded after synthesis before reaching the plasma membrane [29]. In the past decades several approaches were explored to rescue the mutant CFTR, such as secondary rescue mutations were designed to restore the stability and function of the CFTR ΔF508 [30-33]. Similar rescue mutations at homologous positions in ABCG2 may also increase the stability and function of the ABCG2 Q141K protein. Thereby target locations can be identified facilitating to develop drugs for treatment of gout or to design more effective chemotherapeutic agents.

**Objectives**

I aimed to characterize the functional expression of the ABCG2 Q141K protein. My objectives involved the investigation of the role of the Q141 and F142 amino acids in the protein maturation and activity.

During my doctoral research I interrogated the following questions:

- How does the Q141K amino acid alteration affect the structure and function of ABCG2?
- What are the consequences of deleting the F142?
- Are strategies developed for rescuing CFTR ΔF508 able to restore the functional expressions of ABCG2 ΔF142 and Q141K proteins?

**Methods**

1) ABCG2 DNA sequences with mutations were designed by PCR-based side-directed mutagenesis technique and cloned into protein expression vectors.
2) ABCG2 variants were expressed in mammalian (HEK293), insect (Sf9) and bacterial (E.coli) cell lines, as different methods required different expression
systems. The expression levels were analyzed by Western blotting using ABCG2 specific antibodies, and quantified by densitometry.

3) The cellular localization of the proteins in HEK293 cells was studied by confocal microscopy using ABCG2 specific BXP21 antibody recognizing NBD. The ABCG2 cell surface expression was further investigated and quantified by FACS (Fluorescence-activated cell sorting) using ABCG2 specific 5D3 antibody recognizing the 3rd extracellular loop of this transporter.

4) Transport function was measured as the accumulation of fluorescent Hoechst 33342 dye in HEK293 cells, using FACS.

5) ATPase activity was determined by the liberation of inorganic phosphate from ATP with a colorimetric reaction in Sf9 membranes containing ABCG2 variants.

6) Conformation and stability of the ABCG2 variants were studied by (1) the capability of dimer formation in the presence of bifunctional PMPI cross-linker and DTT reducing disulfide bridges, and (2) limited proteolytic assay determining the dynamics of producing and degrading of fragments. These studies were performed in Sf9 membranes.

7) Isolated NBD was produced in E.coli as an MBP fusion protein and purified by affinity chromatography for comparing the ABCG2 variants by CD spectroscopy.

8) The identification and selection of locations for secondary site ABCG2 mutations were performed using data mining and sequence alignments. These in silico methods were adapted to other ABC proteins.

**Summary, thesis**

1) **The Q141K variant exhibits a decreased stability and function compared to the WT protein, while the ΔF142 is a highly unstable and dysfunctional form.** The Q141K protein is expressed at a lower level compared to the WT. The ABCG2 ΔF142 has no mature form and it is present only in intracellular membrane compartments of the cell. Unlike the Q141K variant, which is able to dimerize, the ΔF142 variant does not form a dimer. The WT, Q141K and ΔF142 monomers are stable, but an accelerated degradation of the Q141K NBD and the ΔF142 NBD upon protease treatment can be observed as compared to the WT. The ATPase activity of the Q141K variant is diminished, but substrates and inhibitors can stimulate and inhibit it, respectively. In contrast, the ΔF142 mutant does not show any activity.
2) Isolated, folded ABCG2 NBD with MBP fusion partner can be expressed and purified by affinity chromatography. Based on CD spectroscopy, the isolated NBD has secondary structural elements similar to isolated NBDs of other ABC proteins. Thermal stability experiments by circular dichroism suggest that the unfolding of Q141K NBD differs slightly from that of the WT, while the ΔF142 NBD exhibits a highly denatured structure.

3) The secondary site, G188E amino acid mutation in the NBD increases the expression of the Q141K variant significantly. However, this mutation impairs the transport function and decreases the ATPase activity of ABCG2.

4) The G188E mutation forces the protein into a conformation, in which the extracellular loops are arranged in a conformation similar to that stabilized by the Ko143 inhibitor. The ABCG2 G188E binds the 5D3 antibody as strong as the WT protein in presence of the inhibitor, but it does not occupy the binding site of the inhibitor. Based on limited proteolytic experiments, the G188E and the Ko143 do not alter the stability of the protein.

5) Based on our homology model we claim that the side chains of Q141 and F142 head to opposite directions which suggests that these two amino acids interact with different regions of the protein thus potentially have different roles in providing the functional conformation.

6) We developed the ABCMdb: Database for mutations in ABC proteins web database, which contains most of the published variants and mutated forms of human ABC membrane proteins. The database is available at http://abcm2.hegelab.org.

Conclusion

Based on our homology model and experiments, we suggest that the effects of Q141K are drastically different from the effects of ΔF142. F142 probably forms contacts with different amino acids compared to Q141, and the deletion of Phe makes the protein highly unstable and dysfunctional. A possible explanation of the more pronounced effect of F142 in ABCG2 as compared to that of F508 in CFTR, is that the deletion of F142 in the homodimer affects the interface at two different spatial locations, while ΔF508 expresses its effects only at one point. Our results are consistent with observations in the literature, we showed that the Q141K variant exhibits a decreased function and stability compared to
the WT. Since the Q141 is localized at the surface of the NBD relatively far from the nucleotide binding pocket, the Q141K change influences the ATP hydrolysis allosterically.

The G188E rescue mutation in the Q141K protein increases the protein expression level but reduces the ATPase activity and function. Our experiments revealed that the G188E mutation, similarly to the effect of Ko143 [19, 34], alters the conformation of 3rd extracellular loop, but it does not affect the binding ability of this inhibitor. This observation indicates that the G188E amino acid change in the NBD induces conformational changes also in the TMD.

The ABCMdb web application contains most of the variants and mutant forms of the human ABC membrane proteins, facilitating basic research of these membrane transporters and also developing strategies to rescue disease-causing variants.

**Related publications**


**References**


