# Expression and functional investigation of two less characterized human ABCG proteins: the ABCG1 and ABCG4 transporters

Doctoral Thesis

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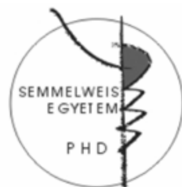


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## INTRODUCTION

ABC (ATP-Binding Cassette) proteins form one of the largest protein families. The members of the family are present in all living organisms from microbes to humans. In humans there have been 48 ABC proteins identified. An ABC protein is defined by the presence of the ATP-binding domain (so called ABC unit) that contains three conserved sequences such as the Walker A, the Walker B and the ABC signature regions. The ABC proteins are modular proteins, they are built from the above mentioned ABC units and transmembrane domains containing usually six membrane helices. A functionally active protein comprises at least two ABC units and two transmembrane domains. In humans, these four domains are present within one polypeptide chain ("full transporters"), or within two separate proteins ("half transporters"). To our current understanding an ABC half transporter becomes functionally active only after dimerization.

With regard to their function, ABC proteins can be classified into three groups: active transporters, which perform uphill transport of substances across the membrane; regulated ion channels; and receptors, which transmit extracellular signals into the cell. All three types of ABC transporters utilize the energy the ATP binding and hydrolysis to accomplish their function.

The human ABCG subfamily consists of five members. They are half-transporters containing only one ABC domain and one transmembrane domain. Some members of the ABCG subfamily form homodimer (ABCG2), while others function as an obligatory heterodimeric complex (ABCG5 and ABCG8). ABCG2 is a well-characterized multidrug-transporter, ubiquitously expressed in different tissues, and presumably plays a key role in the protection against xenobiotics. ABCG5 and

ABCG8 are shown to function as a heterodimeric active transporter for sitosterols. The mutation of either one of these transporters causes a rare inherited disease, sitosterolemia, characterized by increased plasma level of plant sterols and cholesterol, resulting in premature coronary atherosclerosis and cardiac infarction.

Much less is known about the function, localization, and mechanism of action of the human ABCG1 and ABCG4 proteins. At the beginning of our work very little data on these proteins were available. Only their encoding genes and their tissue distribution had been described. It had also been reported that the expression of both ABCG1 and ABCG4 is regulated by cholesterol and lipoproteins in macrophages, suggesting a role for these proteins in lipid metabolism. From sequence analysis became evident that ABCG1 and ABCG4 are the most closely related proteins in the ABCG subfamily, making them good candidates for heterodimerizing counterparts.

## AIMS

Our general goal was to understand the function and the mechanism of action of ABCG1 and ABCG4 proteins. During our work we intended to accomplish the following specific aims:

1. We planned to express the human ABCG1 and ABCG4 proteins in heterologous insect (Sf9) cell system. In addition to the wild type proteins, we intended to generate and express non-functional mutant variants of ABCG1 and ABCG4 in Sf9 cells.



2. We also intended to generate both polyclonal and monoclonal antibodies that specifically recognize ABCG1 and ABCG4 proteins.

3. We planned to perform functional characterization of ABCG1 and ABCG4 proteins by using various *in vitro* methods such as ATPase assay and direct transport measurement. By using these assay systems, we intended to screen a large number of compounds to identify the substances transported by these proteins.

4. We also intended to investigate whether these proteins function as homodimers, as ABCG2 transporter does; or they can form heterodimers, as ABCG5 and ABCG8 proteins do.

## METHODS

The cDNA of the human ABCG1 and ABCG4 was cloned into the pAcUW21L transfer vector, then Sf9 cells were transfected and the baculovirus containing supernatants were harvested. The potentially inactive mutant variants containing a missense mutation in a highly conserved motif of the catalytic site were generated by PCR mutagenesis, and also cloned into the transfer vector. For functional studies, Sf9 cells were infected with the baculoviruses and isolated membranes containing the different variants of the human ABCG1 and ABCG4 proteins were prepared.

In order to elicit polyclonal and monoclonal antibodies that distinguish between the human ABCG1 and ABCG4 proteins, GST fusion proteins containing the intracellular part of either

ABCG1 or ABCG4 was prepared and purified. Polyclonal sera from mice immunized with the GST fusion proteins were screen for specific recognition, and hybridomas were generated. The selective and sensitive monoclonal antibodies were identified by Western blot analysis.

The functional characterization of the studied proteins was carried out by using ATPase assay and direct transport measurement. The vanadate sensitive ATPase activity of membrane preparations containing the ABCG1 or ABCG4 proteins was assessed by measuring the phosphate liberation. To identify potential substrates and inhibitors, we screened a compound library consisting of more than 100 substances by using the ATPase assay system.

In the transport measurements, we examined the accumulation of fluorescent compounds that stimulated the ATPase activity. Their uptake in living Sf9 cells or isolated inside-out membrane vesicles was detected by flow cytometry. Both wild type and mutant variants were investigated in these assays in the absence and presence of the identified inhibitors.

The effect of ABCG1 and ABCG4 proteins on the Sf9 cell viability was investigated by the determined by counting the dead and living cells by using trypan blue staining at certain time points after infection.

The possible interaction between ABCG1 and ABCG4 proteins was investigated by using membrane preparations isolated from Sf9 cells co-expressing wild type and mutant proteins in various combinations. Both the basal and the substrate-stimulated ATPase activity of these membrane preparations were examined.



## RESULTS

The human ABCG1 and ABCG4 proteins are the most closely related proteins within the ABCG subfamily; they share 72% amino acid identity. We performed a membrane topology modeling by an online available software program (http://www.enzim.hu/hmmtop/), which indicated that these proteins comprise only one ABC and one transmembrane domain, and the nucleotide binding domain is located N-terminally to the transmembrane domain similar to the predicted domain structure of other members of the ABCG subfamily.

For functional characterization of human ABCG1 and ABCG4 proteins we utilized the baculovirus-Sf9 cell heterologous expression system, which was successfully used previously to investigate other ABC proteins including the ABCG2 transporter. First we cloned the cDNA of human ABCG1 and ABCG4 into the transfer vector and expressed the proteins in Sf9 cells. We also produced catalytic site mutant variants of both transporters by replacing the conserved lysine residue in the Walker A region with metionine ($ABCG1_{K124M}$, and $ABCG4_{K108M}$, respectively). This mutation was expected to abolish the activity of the transporter, as it was previously observed in the case of ABCG2 and other ABC proteins. Thus, the catalytic site mutant variants can serve as negative controls for the functional characterization of the transporters. To determine the background ATPase activity of Sf9 membranes we produced membrane preparations from cells expressing β-galactosidase. As a positive control, we used a variant of ABCG2 ($ABCG2_{R482G}$), which is well characterized in assay methods we applied. The inactive counterpart of this ABCG2 variant, $ABCG2_{R482G,K86M}$ served as a negative control in our studies. We expressed all these variants in Sf9 insect cells, and prepared isolated membranes. Both ABCG1 and ABCG4

proteins were present in the membrane preparations at high level and detectable by Comassie staining after gel electrophoresis.

A crucial point of our project was eliciting selective antibodies against these very closely related proteins to make possible their sensitive and specific detection. We managed to generate both polyclonal and monoclonal antibodies against both ABCG1 and ABCG4. These antibodies proved to be specific and distinguish between the two transporters. Both polyclonal and monoclonal anti-G1 antibodies were suitable for Western blot analysis, and recognized ABCG1 and $ABCG1_{K124M}$ but not other ABCG proteins or any proteins of the Sf9 membrane. Similarly, the ABCG4 and $ABCG4_{K108M}$ proteins were specifically recognized by anti-G4 polyclonal and monoclonal antibodies. Both ABCG1 and ABCG4 proteins were observed at approximately 60 kDa molecular weight, which correlates with the expected size. The generated antibodies were proven to be suitable also for immunostaining, and allowed us sensitive and selective detection of the studies proteins.

The function of the human ABCG1 and ABCG4 proteins expressed in insect cells were investigated by various *in vitro* methods including ATPase assay and direct transport measurements. Most ABC transporters bind and hydrolyze ATP and use the acquired energy for active transport. Numerous previous studies demonstrated that the function of many ABC transporters can be monitored by the ortovanadate-sensitive ATPase activity in isolated Sf9 membranes containing the studied transporter in a large amount. When we examined the human ABCG1 protein a relatively high vanadate-sensitive ATPase activity was detected compared to the background. This activity was completely abolished in the $ABCG1_{K124M}$ variant, indica ting the generated mutation in the catalytic site resulted in



an inactive protein, as it was aimed. Similarly, ABCG4 exhibited some ATPase activity, and the $ABCG4_{K108M}$ mutant was found to be inactive. The ATPase activity of wild type ABCG4 was lower than that of ABCG1, but still detectable over background.

Since the stimulation of the ATPase activity is characteristic for the transported substrates, whereas inhibitors of the transporters block the ATPase activity, to identify potential substrates and inhibitors, we assessed the effect of a large number of compounds on the ATPase activity of the studied proteins. We screened about 100 substances of different origin, which compounds previously identified as substrates or inhibitors of other ABC transporter. These included anticancer agents, receptor agonists, ion-channel modifiers, hormones, neurotransmitters, conjugated bile acids, sterol derivates, etc. Screening of this compound library revealed a stimulatory effect of two fluorescent indicators on the ATPase activity of ABCG1. Rhodamine 123 and rhodamine 6G stimulated the ABCG1-driven ATPase activity in a concentration dependent manner with a 2-fold and 1.5-fold maximum activation, respectively, as compared to the basal activity. No stimulation by these compounds was observed in the $ABCG1_{K124M}$-containing membrane preparations. Surprisingly, neither of the tested compounds stimulated the ATPase activity of ABCG4.

By using this screening assay method we were able to identify several inhibitors of the ABCG1 function. The ion-channel blocker benzamil, the immunosuppressant cyclosporine A, the sex hormone progesterone, as well as the thyroid hormones L-thyroxin and triiodo-thyronine inhibited both the basal and the rhodamine 123-stimulated ATPase activity of ABCG1. The minor ATPase activity of ABCG4 did not allow us to identify inhibitors.

The ATPase assay provides a rapid screening method for investigating the interaction of a tested compound with the studied proteins. Transport measurement offers an alternative, direct assessment of the function of these transporters. Since rhodamine compounds are easily detectable, fluorescent dies, we examined the ABCG1-dependent rhodamine transport in two different fluorescence based methods. One of these techniques is the assessment of dye accumulation in intact cells, which was previously developed and successfully applied to investigate the transport function of other ABC proteins including the ABCG2 transporter. We adopted this method to study the transport properties of ABCG1 protein. In addition, we developed a novel functional assay method in which we measured the fluorescent dye uptake in membrane vesicles containing the studies transporter. Although ABCG2 mediated rhodamine transport was nicely detected by both method, we could not observe ABCG1-mediated rhodamine transport. A plausible explanation for this observation is that ABCG2 with its substantial transport capacity can generate a concentration gradient against the passive leakage of rhodamine, which is a membrane permeable compound; whereas ABCG1 possesses only a low transport capacity, which can not exceed the diffusion-driven leakage, thus, transport activity of ABCG1 remains undetectable by this method.

In addition to the primary observations, these transport measurements drove our attention to a phenomenon that served as a base for subsequent studies. We observed that the overexpression of ABCG1 results in cell death. When Sf9 cells were infected with baculovirus containing the wild type ABCG1 sequence, killing of the cells occurred significantly earlier than that of cells infected either with β-galactosidase, ABCG2, or the $ABCG1_{K124M}$ variant. Similarly, the wild type ABCG4



accelerated cell death in baculovirus-transfected Sf9 cultures, whereas the $ABCG4_{K108M}$ had no effect on the cell killing kinetics. These observations indicated a cytotoxic effect of the ABCG1 and ABCG4 transport activity, which hypothesis was subsequently verified by detailed studies.

As mentioned above, the members of ABCG subfamily are half-transporters, and form either homodimers (ABCG2) or heterodimers (ABCG5 and ABCG8). One of our aims was to investigate dimerization of ABCG1 and ABCG4. Our observation that either ABCG1 or ABCG4 exhibited substantial ATPase activity, when expressed alone, suggests that these half transporters can function as homodimers. However, there are several observations that make feasible to hypothesize heterodimerization between these half transporters. These include their extreme sequence similarity, their similar transcriptional regulation, and the fact that their *Drosophila* orthologs function as heterodimers. To study the possible heterodimer formation, we co-expressed the wild type and the inactive mutant ABCG proteins in different combinations, and investigated the ATPase activity of the isolated Sf9 membranes. As controls, membrane preparations containing either ABCG1 or ABCG4 alone were used. The expressions of the studied proteins were thoroughly adjusted to a similar level. Co-expression of the wild type ABCG1 and ABCG4 did not alter the ATPase activity as compared to the membranes contained ABCG1 alone. However, the ATPase activity completely abolished when wild type ABCG1 was co-expresses with the inactive $ABCG4_{K108M}$. The specificity of this inhibitory effect was demonstrated by the observation that the mutant $ABCG2_{K86M}$ had no effect on the ATPase activity of ABCG1, and reversely, the inactive $ABCG4_{K108M}$ did not influence the ATPase activity of ABCG2.

Taken together, these functional analyses suggest a specific interaction between the ABCG1 and ABCG4 proteins.

## CONCLUSIONS

For studying ABCG1 and ABCG4 proteins, we adopted the methods that were previously used for the characterization of other ABC transporters. These include the baculovirus-Sf9 expression system, the ATPase measurement, and the direct transport assay using fluorescent dyes. In addition to the basic characterization, our studies revealed some peculiarity of these transporters. ABCG1 exhibited a relatively high basal ATPase activity, which is not typical for most ABC transporters, although ABCG2 also possesses similar high basal ATPase activity. Surprisingly, most compounds that are substrates of other ABC transporters and stimulate their ATPase activity had no effect on the activity of ABCG1 and ABCG4. From a large number of tested compounds only two rhodamine dyes stimulated the ATPase activity of ABCG1. Although the physiological substrates of these transporters still remain to be identified, the basal ATPase activity shown by both proteins suggests that one or more endogenous substrates are present in the membrane preparation. This hypothesis is in good accordance with previous findings proposing an involvement of these proteins in the lipid homeostasis, thus, the putative endogenous substrate(s) can be substance(s) of lipid character. In addition to the detailed analysis of ATPase and transport activity of ABCG1 and ABCG4, our studies elucidated the cytotoxic effect of these proteins. This characteristic is a unique feature, and has not been described in connection with ABC transporters yet.



We also aimed to investigate the issue of dimerization. Our studies revealed that ABCG1 and ABCG4 can function as homodimers but can also form heterodimers. Since their tissue distribution shows only partial overlapping, we can speculate that these transporters function as heterodimers in tissues where both are expressed, while they act as homodimers in other tissues.

Taken together, our work is one of the first studies in expression and functional characterization of the human ABCG1 and ABCG4 proteins, which are thought to be important participants in lipid metabolism, and atherogenesis. Thus, our results can serve as a basis for further studies on lipid and atherosclerosis research.

**Publications in connection with the thesis:**

Functional expression and characterization of the human ABCG1 and ABCG4 proteins: indications for heterodimerization.

Cserepes J, Szentpetery Z, Seres L, Ozvegy-Laczka C, Langmann T, Schmitz G, Glavinas H, Klein I, Homolya L, Varadi A, Sarkadi B, Elkind NB.

Biochem Biophys Res Commun. 2004 Jul 30;320(3):860-7.

Seres L; Cserepes J; Elkind NB; Töröcsik D; Nagy L; Sarkadi B; Homolya L.

Functional ABCG1 expression induces apoptosis in macrophages and other cell types

BBA – Biomembranes (accepted for publication)

**Other publications:**

The role of ABC Transporters in Drug Resistance, Metabolism, and Toxicity

Hristos Glavinas, Péter Krajcsi, Judit Cserepes, and Balázs Sarkadi

Current Drug Delivery, 2004, 1, 27-42

Tyrosine kinase inhibitor resistance in cancer: role of ABC multidrug transporters

Csilla Ozvegy-Laczka, Judit Cserepes, N. Barry Elkind, Balazs Sarkadi

Drug Resistance Updates 8 (2005) 15–26.