From genetic disease to protein structure: Analysis of disease associated missense mutations of the ABCC6 transporter

Theses of PhD dissertation

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Introduction

Human ABC (ATP Binding Cassette) proteins are ATP utilizing transmembrane transporters that pumps substrates across the plasmamembrane and intracellular membranes. The functional core unit of ABC transporters consists of two ABC domains and two transmembrane domains. The ABC domains are organised in head to tail orientation to form two composite catalytic sites that close upon ATP binding. The two nucleotide-binding pockets are formed by Walker-A and Walker-B motifs of one nucleotide binding fold (ABC domain) and by the signature motif of the opposite nucleotide-binding fold. Transmembrane domains that form the substrate translocation pore are connected to the catalytic ABCs through rigid helices that transmit conformational changes generalized during the ATP-cycle.

Mutations within the *ABCC6* gene are the genetic basis of two heritable disorders related to soft tissue calcification: pseudoxanthoma elasticum (PXE) and generalized arterial calcification of infancy (GACI). Pseudoxanthoma elasticum is a recessive genetic disorder characterized by progressive calcification of elastic fibers in the skin, in the medial layer of middle-sized arteries and within the Bruch's membrane of the retina. Most severe clinical consequences of the disease are the risk of cardiovascular events and the macular degeneration leading to loss of visual acuity. While PXE is underlined by high phenotypic variability; i.e. milder forms of the disease often remain undiagnosed even for lifetime; generalized arterial calcification of infancy is a more severe disorder characterized by the calcification of the lamina media of large and medium sized arteries that is associated with the proliferation of the intimal layer, leading to stenosis of the vessels and myocardial ischemia within the first months of life. Since 2000, when mutations at the *ABCC6* locus were identified as the genetic background of PXE, more than 400 sequence variations affecting this region were published. Despite the large number of *ABCC6* mutations, identified thus far, no clear genotype-phenotype correlation has emerged.

The ABCC6 transporter has also been extensively studied since than. Tissue distribution and subcellular localization experiments revealed marked liver expression with exclusive basolateral localization and with no or extremely low within tissues affected by the disease, suggesting a role for ABCC6 in the sinusoidal efflux of an unidentified metabolite. According to this PXE was hypothesized as a metabolic disorder. However, the

physiologic substrate of ABCC6 and the pathological background of the disease still remain unknown.

Patients with a single gene Mendalian disorder, the PXE-like syndrome, underlined by mutations in the gamma-glutamyl carboxylase (GGCX) gene, also develop cutaneous signs similar to PXE. The GGCX enzyme is responsible for the secondary modification, thus the activation of Ca-chelating proteins. These include vitamin-K dependent coagulation factors and proteins participating in anti-calcification processes of connective tissues. This suggested that while the defective GGCX enzyme could be responsible for the hepatic and peripheral manifestation of PXE-like syndrome the lack of vitamin-K compounds at peripheral tissues would be the basis of ABCC6 associated calcification processes in case of PXE and the ABCC6 related GACI cases. According to this hypothesis ABCC6 would be the transporter of (a) vitamin-K form or derivative utilized in peripheral tissues.

Aims

In one part of my PhD period I focused on the functional properties of the transporter and investigated its potential involvement in the Vitamin K transport.

The other part of my work aims to analyze disease-associated mutations of the *ABCC6* gene and those of the transporter.

My specific aims were as follows:

1. To synthesize and to purify the radioactively labeled and the unlabeled GSH conjugated form of the VitK3 metabolite (VK3GS).

   To test ABCC6 transporter and additional hepatic ABC transporters, ABCC1, ABCC2, ABCC3, and ABCG2, for their ability to transport the VK3GS conjugate in *in vitro* vesicular transport measurements. I aimed to determine whether ABCC6 or any of the above hepatic ABC proteins is a transporter of the VK3GS conjugate.
2. Since there were already hundreds of disease-causing mutations described in the literature affecting the *ABCC6* gene thus far we decided to establish a validated database of the sequence variations.
3. In order to recall structure and function data embedded amongst the numerous disease-associated mutations we aimed to generate three dimensional homology models of the ABCC6 protein.

4. In order to analyze the consequences of disease-associated mutations of the ABCC6 transporter *in vitro* I have selected missense mutations that are frequent in PXE and affect conserved functional or structural surfaces of the protein. I aimed to investigate their functional properties *in vitro* in vesicular transport measurements and to investigate their subcellular localization *in vitro* in MDCKII mammalian cells. As part of a collaborative work our specific aim was to identify transport competent but mislocalized mutants. These may serve as potential candidates in pharmacological rescue experiments targeting stability/folding correction of disease associated missense mutants both *in vitro* and *in vivo*.

Methods

Data processing of ABCC6 database

Published sequence variants of the *ABCC6* gene were collected from papers available in the PubMed database. The online database is powered by an LOVD platform; it was created in a collaborative work with PXE International and it operates on the server of the NCBI.

Model building

Protein sequence alignments of ABCC6, Sav1866, HlyB–ABC, CFTR–ABC and P-gp proteins were generated by ClustalW2 program using the default settings. Homology model building was performed by using the Sav1866 bacterial ABC transporter coordinates (PDB Accession No: 2ONJ 3.4Å) as well as the coordinates of the HlyB ABC–ABC dimer with ATP-Mg and those of the N-ABC CFTR homodimer (PDB Accession No: 1XEF 2.5Å, 2PZE 1.7Å, respectively) in case of the closed conformation (outward facing) model and the coordinates of the mouse P-gp (PDB Accession No: 3G5U 3.8Å) in case of the open conformation model. Two hundred structures were generated using the Modeller 9.3 software package. The one with the lowest objective function of the 200 generated models were used in the present study. Figures were made with PyMOL software. Fisher exact test was used for statistical calculations.

DNA constructs of ABCC6 variants

p.S1121W, p.T1301I, p.Q1347H and p.R1459C mutants were genereted by overlap extension mutagensis PCR method in pAcUw21L baculovirus transfer vector. These constructs were used to express ABCC6 mutants in Sf9 (Spodoptera frugiperda) insect cells. The cDNA cassettes harbouring the above PXE-associated missense mutations were

subcloned into SpSldS retroviral vector and pLIVE vector that were used for *in vitro* expression in MDCKII cells and for *in vivo* experiments in mice, respectively.

Cell culturing

Sf9 insect cells were maintained at 27°C in TNM-FH medium supplied with FBS, penicillin and streptomycin. Phoenix-Ampho and MDCKII cells were cultured in humidified CO_2 thermostate at 37°C, in DMEM medium supplied with FBS, penicillin and streptomycin.

Expression of S1121W, T1301I, Q1347H and R1459C variants in Sf9 cells

Sf9 (Spodoptera frugiperda) insect cells were co-transfected with linearized baculovirus vector and pAcUw21L vector containing ABCC6 wt or the mutant cDNA constructs, as recommended by the manufacturer (BaculoGold kit (BD Biosciences Pharmingen). Viral-supernatants corresponding to the highest expression levels were cloned with end point dilution method and $3x10^7$ cell were transfected with the amplified virus. Cells overexpressing recombinant proteins were harvested after 72 hours.

Membrane preparation and the determination of membrane protein content

After washing and homogenization steps using Potter-Elvehjem tissue grinders (Wheaton) membrane fraction of the cell was isolated with differential ultracentrifugation at 60.000G. Membrane pellet was suspended to obtain approximately 5-10 mg/ml total protein concentration and subjected for final homogenization. Aliquots of membrane preparation samples were stored at -70°C. Total protein content of the membrane preparation samples was measured with the *modified Lowry-method*. The relative amount of uptake-competent inside-out vesicles in different membrane preparation samples was estimated on the bases of ^{45}Ca uptake by endogenous Ca-transporters in Sf9 cells.

Leammli SDS-polyacrylamide gel electrophoresis and immunoblot

Membrane preparation and cell lysate samples were diluted in disaggregating buffer and loaded on 6-7.5% polyacrylamide gel with Protean electrophoresis equipment (Bio-Rad). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes by standard electro-blotting with Mini-Trans-Blot or by semidry blot equipments according to the manufacturer's protocols (Bio-Rad). ABCC6 wt and mutant proteins were detected with HB6 polyclonal or M6II-7 monoclonal antibodies. HRP-conjugated secondary antibodies and a chemiluminescent (ECL) imaging (GE Healthcare Life Sciences) was used to develop signals.

Synthesis and purification of the [^{3}H]K3GS conjugate.

280µM [^{3}H]GSH was rapidly added to 0.1M K3 solved in ethanol. The reaction was incubated on 4^{o}C overnight in silanized glass tubes. [^{3}H]K3GS crystals were vacuum dried, solved in 5% trifluoric acetate and purified by HPLC. Unlabeled VK3GS has been synthesized similarly and purified by chrystallization and chloroform-washing. The chemical identity of the compounds were verified by HPLC-MS. VK3GS solutions were stored at -20^{o}C in dark glass vials and used within 30 days.

[^{3}H]LTC4, [^{3}H]E_2-17G, [^{3}H]MTX and [^{3}H]K3GS Transport measurements

Measurements were performed with rapid filtration method. ATP-dependent transport was calculated from data measured in the presence and absence of 4 mM MgATP. Radioactivity was detected in OptiFluor scintillation cocktail (Perkin Elmer) with a scintillation counter (Wallac 1409 DSA). Transport kinetics were calculated and visualized with KaleidaGraph (Synergy) Software.

Retroviral expression of ABCC6 mutants in MDCKII cells

Phoenix-Ampho "packaging" cells/well were co-transfected with the recombinant retroviral vector pSpSldS containing the wild type or mutant constructs of ABCC6 cDNA parallel with helper vectors expressing M57 and GALV viral proteins. Transfection was performed using the calcium phosphate precipitation method, according to the manufacturers protocol (GIBCO). Virus containing supernatant was collected and MDCKII cells were transduced with the previously harvested recombinant retroviruses. Polybrene was also added in a final concentration of 6 mg/ml. After centrifugation (1000G, 90min) cells were maintained at 37°C in humidified, 5% CO_2 incubator.

Immunostaining of MDCKII cells and confocal imaging

In order to obtain nonpolarized cell culture MDCKII cells were maintained on 8-well chambers (coverslips) and subjected for immunostaining after two days. To obtain polarized cultures MDCKII cells were maintained on Transwells and subjected for immunostaining 8-10 days after reaching confluency.

Before immunostaining cells were washed in Dulbecco's Phosphate Buffered Saline, fixed with 4% paraformaldehide and treated with pre-cooled methanol for 5 min. After blocking, samples were incubated with primary and secondary antibodies solved in blocking buffer for 1 hour. Nuclei were stained with DAPI if it was indicated and samples were subjected for confocal microscopy.

Confocal imaging of immunostained MDCKII samples was performed using Olympus IX-81/FV500 laser scanning confocal microscope; images were analyzed by Olympus FluoView 4.7 software.

Results

1. I have synthesized and purified unlabeled and radioactively labelled VK3GS conjugate from VitK3 and GSH or [^{3}H]GSH, respectively.
2. I have investigated the ATP-dependent VK3GS transport of ABCC6, ABCC1, ABCC2, ABCC3 and ABCG2 transporters on validated membrane vesicles. In case of ABCC6 transporter I could not detect significant VK3GS-transport, indicating that ABCC6 is most probably not involved in the export of VK3GS from hepathocytes [Fülöp et al., 2011].
3. In similar experimental conditions ABCC1 transporter was proved to be a high capacity transporter of VK3GS, with approximate K_M and V_{max} values of 1.45μM and 240 pmol/mg membrane protein/min, respectively [Fülöp et al., 2011]. This transport was specific, as 500nM of LTC4, the physiological substrate of ABCC1, and the common MRP-inhibitor, MK571 (10μM) inhibited the transport to a residual activity of 35% and 23%, respectively. No ATP dependent transport of the VK3GS compound was observed in case of the ABCC1 inactive mutant G771D.
4. These assays also revealed ABCC2 as a potential low capacity transporter of the VK3GS conjugate, with a characteristic "co-transport" mechanism.
5. No transport of VK3GS by ABCC3 and ABCG2 transporters could be detected amongst similar conditions in our transport system.

6. I have contributed to the establishment of a validated database collecting all the PXE and GACI related *ABCC6* mutations that has been published in the literature. Most frequently used information, i.e.: the sequence variation at DNA level; the sequence variation at protein level; the status: mutation/polymorphism; the type of alteration; the genomic position of the alteration; the genomic region affected; the protein region affected; in case of a "C>T" variation if it has occurred at a "5'CpG", is listed in the table. All publications that report on a particular sequence variation are given. In every case where it was available allele frequency data is

also indicated. These data served as the major basis of the online "ABCC6 LOVD" database (`http://www.ncbi.nlm.nih.gov/lovd/home.php?select_db=ABCC6`).

7. I have also analyzed the CG content of the 4512 nucleotide long cDNA as well as the distribution of CpG dinucleotides within the coding region. We concluded that the distribution of single nucleotide substitutions within the coding sequence is not random, CpGs are more frequently affected. 29% of the single nucleotide substitutions affecting the coding region occurred on 312 bases (156 5'CpG sites) that correspond to the 6,9 % of the coding nucleotides.
8. I have also shown that mutational events affecting CpG dinucleotides largely contribute to the number of disease causing mutations in PXE and GACI diseases. Mutations identified on CpG sites along the *ABCC6* gene represent the 20.5 % of all disease causing mutations (220), based on the data reported until the end of 2013.

9. I have contributed to the generation of homology models representing the inward-facing and the outward-facing conformations of the ABCC6 protein. These models were generated on the basis of the crystal coordinates of the Sav1866 (PDB ID: 2ONJ, 3.4Å) and those of the mouse Abcb1 (PDB ID: 3G5U) transporters.
10. We analyzed the distribution of disease-associated missense mutations on predicted functional and structural surfaces of the protein and detected the significant clustering of missense mutations on the ABC-ABC and the ABC-ICL contact surfaces [Fulop et al., 2009]. These findings are considered as the first genetic proof of the essential role of these domain-domain interactions. I have also investigated and excluded the potential role of DNA structure-related mutational hot spots in the observed clustering.
11. In contrast to the results of Kelly et al., 2010 I questioned the predictive value of homology models, e.g. if homology models are useful in forecasting pathological consequences of individual amino acid replacements in the protein structure.

12. I have investigated the structure and function consequences of mutations affecting the protein structure by expressing and analyzing four disease-associated missense mutants: S1121W, T1301I, Q1347H and R1459C, of the ABCC6 protein *in vitro*. I

have identified them as transport active but mis-localized variants [Pomozi, Brampton, Fülöp, Chen, Apana, Li, Uitto, LeSaux and Váradi, 2014].

Discussion

ABCC6 is thought to transport (an) unidentified substrate(s) that directly or indirectly prevents ectopic soft tissue calcification in organs affected by PXE and GACI diseases. Based on *in vitro* transport experiments I disproved a recent hypothesis suggesting a Vitamin K metabolite, the VK3GS conjugate, as potential physiological substrate of the ABCC6 transporter. *In vitro* and *in vivo* data of others also supported my results, thus I concluded that ABCC6 protein is not involved in the elimination of VK3GS conjugate from hepathocytes.

Since 2000, when mutations at the *ABCC6* locus were identified as the genetic background of PXE, more than 400 sequence variations affecting this region were published. As it is impossible to characterize all the protein variants using regular *in vitro* or *in vivo* methods, the development of appropriate *in silico* approaches focusing on structural and functional consequences of sequence variations have great importance. Validated databases, collecting published experimental evidences related to mutations as well as clinical phenotype data of affected patients are also extremely helpful.

The collection of ABCC6 variants, generated in Budapest by Orsolya Symmons and me, served as the major basis in establishing the online database (`http://www.ncbi.nlm.nih.gov/lovd/home.php?select_db=ABCC6`).

I have also contributed to the generation of homology models of ABCC6 protein. The homology model of ABCC6 [Fulop et al, 2009], also linked to the online database, gives an opportunity to answer structural and/or functional consequences of mutations via embedding missense mutations in a structural view of the protein. Our observation that disease associated ABCC6 mutations show significant clustering on the ABC-ABC and ABC-ICL contact surfaces in the predicted structure was the first genetic proof supporting the fundamental role of the ABC-ICL intramolecular surfaces.

In vitro cell free activity assays as well as *in vitro* and *in vivo* cellular models expressing recombinant proteins are basic experimental systems that may lead to the better understanding of the molecular mechanisms of a protein. As part of a large collaborative work I characterized the transport function and the subcellular targeting of four disease-

associated missense mutants, S1121W, T1301I, Q1347H and R1459C, of the ABCC6 protein *in vitro*. I have identified them as transport active but mis-localized variants. These mutants served as proper candidates for *in vitr*o and *in viv*o folding correction experiments. Results of these experiments may serve as the first steps toward allele specific therapy in PXE and GACI diseases in which no efficient therapy exists.

The candidate's publications:

1. **Fülöp K**, Barna L, Symmons O, Závodszky P, Váradi A.: Clustering of disease-causing mutations on the domain-domain interfaces of ABCC6. Biochem Biophys Res Commun. 2009 Feb 13; 379(3): 706-9.

2. Váradi A, Szabó Z, Pomozi V, de Boussac H, **Fülöp K**, Arányi T.: ABCC6 as a target in pseudoxanthoma elasticum. Curr Drug Targets. 2011 May; 12(5): 671-82. Review.

3. Arányi T, **Fülöp K**, Symmons O, Pomozi V, Váradi A.: Predictable difficulty or difficulty to predict. Protein Sci. 2011 Jan; 20(1):1-3.

4. Le Saux O, **Fülöp K**, Yamaguchi Y, Iliás A, Szabó Z, Brampton CN, Pomozi V, Huszár K, Arányi T, Váradi A.: Expression and in vivo rescue of human ABCC6 disease-causing mutants in mouse liver. PLoS One. 2011; 6(9): e24738.

5. **Fülöp K**, Jiang Q, Wetering KV, Pomozi V, Szabó PT, Arányi T, Sarkadi B, Borst P, Uitto J, Váradi A.: ABCC6 does not transport vitamin K3-glutathione conjugate from the liver: relevance to pathomechanisms of pseudoxanthoma elasticum. Biochem Biophys Res Commun. 2011 Nov 25; 415(3): 468-71. 2.

6. Arányi T, Bacquet C, de Boussac H, Ratajewski M, Pomozi V, **Fülöp K**, Brampton CN, Pulaski L, Le Saux O, Váradi A.: Transcriptional regulation of the ABCC6 gene and the background of impaired function of missense disease-causing mutations. Front Genet. 2013 Mar 11; 4:27.

7. Pomozi V, Brampton C, **Fülöp K**, Chen LH, Apana A, Li Q, Uitto J, Le Saux O, Váradi A.: Analysis of pseudoxanthoma elasticum-causing missense mutants of ABCC6 in vivo; pharmacological correction of the mislocalized proteins. J Invest Dermatol. 2014 Apr; 134(4): 946-53.

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