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Functional characterization of rainbow trout (*Oncorhynchus mykiss*) Abcg2a (Bcrp) transporter

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ABSTRACT

ABCG2 (BCRP – breast cancer resistance protein) belongs to the ATP-binding cassette (ABC) superfamily. It plays an important role in the disposition and elimination of xeno- and endobiotics and/or their metabolites in mammals. Likewise, the protective role of ABC transporters, including Abcg2, has been reported for aquatic organisms. In our previous study we have cloned the full gene sequence of rainbow trout (*Oncorhynchus mykiss*) Abcg2a and showed its high expression in liver and primary hepatocytes. Based on those insights, the main goal of this study was to perform a detailed functional characterization of trout Abcg2a using insect ovary cells (*Spodoptera frugiperda*, Sf9) as a heterologous expression system. Membrane vesicles preparations from Sf9 cells were used for the ATPase assay determinations and basic biochemical properties of fish Abcg2a versus human ABCG2 have been compared. A series of 39 physiologically and/or environmentally relevant substances was then tested on interaction with trout Abcg2a and human ABCG2. Correlation analysis reveals highly similar pattern of activation and inhibition. Significant activation of trout Abcg2a ATPase was observed for prazosin, doxorubicine, sildenafil, furosemid, propranolol, fenofibrate and pheophorbide. Pesticides showed either a weak activation (malathione) or strong (endosulfan) to weak (chlorpyrifos, fenoxycarb, DDE) inhibition of trout Abcg2a ATPase while the highest activation was obtained for benzo(a)pyrene, curcumine and testosterone. In conclusion, data from this study offer the first characterization of fish Abcg2a, reveal potent interactors among physiologically or environmentally relevant substances and point to similarities regarding strengths and interactor preferences between human ABCG2 and fish Abcg2a.

1. Introduction

The human ABCG2 (BCRP – breast cancer resistance protein) belongs to the ATP-binding cassette (ABC) transporters superfamily and plays an important role in the elimination of xenobiotics. Together with the P-glycoprotein (Pgp; ABCB1) and members of the MRP (ABCC) subfamily, ABCG2 is a major mediator of the multidrug resistance (MDR) phenotype in tumor cells. Unlike ABCB1 or ABCCs, however, ABCG2 is a 72-kDa half-transporter, consisting of one nucleotide binding domain (NBD) and one membrane spanning domain (TMD) containing 6 α-helices (Wakabayashi et al., 2006). Upon dimerization, two ABCG2 monomers form the functional transporter that is similar to structure and transport mechanism of ABCB1 (Ni et al., 2010; Rosenberg et al., 2015). The binding of a substrate from the inner membrane leaflet or the cytoplasm results in conformational changes, activation and dimerization of two NBDs. Dimerization causes structural changes leading to the outward facing conformation and release of the substrate in the extracellular space. Finally, ATP hydrolysis resets the protein in the inward facing conformation making it ready for a new catalytic cycle (Rosenberg et al., 2015).

Through the described mechanism ABCG2 transports a wide range of structurally diverse xenobiotics, including both positively and negatively charged molecules of relatively high molecular mass and amphiphilic character (Basseville et al., 2014). Similar to ABCB1, ABCG2 is involved in efflux and resistance to many unmodified anticancer drugs (mitoxantrone, topotecan, irinotecan, etoposide, flavopiridol, methotrexate) as well as other therapeutics (statins, sulfasalazine, nitrofurantoin, cimetidine, imatinib). Contrary to ABCB1 and more similar to ABCCs, ABCG2 mediates transport of many conjugated endo- and xeno- biotics (Basseville et al., 2014; Chen et al., 2016). However, unlike ABCCs which are mainly involved in the transport of glutathione and glucuronide conjugates, ABCG2 exhibits preference toward sulfate conjugates (Álvarez et al., 2011; van de Wetering and Sapthu, 2012). Recent findings also support the role of ABCG2 in the cellular homeostasis of porphyrins and related compounds, and consequently in oxidative stress response (Krishnamurthy and Schuetz, 2011).

ABCG2 is expressed in the apical membranes of polarized epithelial cells of different organs involved in absorption (small intestine),

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distribution (placenta and blood brain barrier) and elimination (liver) (Fetsch et al., 2006). Taken together, the tissue distribution, cellular localization, and substrates' specificity of ABCG2 all indicate that this transporter plays a major role in the disposition and elimination of xeno- and endobiotics and/or their metabolites in mammals, together with ABCB1 and ABCC2.

In the context of environmental toxicology, protective role of ABC proteins in aquatic organisms was first recognized in early 1990s, revealing the presence of the Pgp (ABCB) family of transporters in marine and freshwater bivalves. Since then, the presence of the ABCB1 and ABCC types of efflux transporters has been demonstrated in many aquatic species (Bard, 2000; Sturm and Segner, 2005; Luckenbach et al., 2014). Most of the studies have been focused on identification of ABCs at the transcript, protein and/or functional levels, using functional in vivo studies or studies in permanent cell lines or in primary fish cell cultures (reviewed in Luckenbach et al., 2014). Furthermore, those studies essentially utilized multitransporter systems that express many transport proteins with overlapping substrate specificities. Consequently, such systems do not enable a detailed and reliable characterization of single ABC transporter(s). Yet, despite the presumable role of these transporters in defense against natural and man-made substances in aquatic organisms, with the exception of the study done by Fischer et al. (2013) on zebrafish Abcb4, no study has been focused on thorough functional characterization of ABC transporters in heterologous expression systems.

Insect ovary cells (*Spodoptera frugiperda*, Sf9) are the expression system often used in studying mammalian ABC transporters (Meyer et al., 1994; Trometer and Falson, 2010). Due to the high protein expression, membrane vesicles preparation from Sf9 cells overexpressing the target ABC transporter are used for ATPase assays or vesicular transport assays directed to the determination of substrate and inhibitor specificity of ABC transporters, including transporters from the ABCG family (Özvegy et al., 2001; Müller et al., 2006; Pozza et al., 2010). In our previous study (Zaja et al., 2008) we have cloned the full gene sequence of rainbow trout (*Oncorhynchus mykiss*) Abcg2 and showed its high expression in trout liver and primary hepatocytes as well as in other trout tissues. However, recent availability of the full genome data from multiple fish species reveals that there are two Abcg2 genes (*abcg2a* and *abcg2d*) present in the trout genome, and four predicted Abcg2 genes in the zebrafish genome, contrary to mammals that have only one ABCG2 protein. Multiple alignment of our trout sequence with mammalian ABCG2 and zebrafish Abcg2 proteins has revealed a high degree of similarity to mammalian ABCG2 and zebrafish Abcg2a, respectively. Therefore, based on those initial insights, the main goal of the present study was to perform a detailed functional characterization of trout Abcg2a using Sf9 insect cells as a heterologous expression system. Furthermore, basic biochemical properties and substrates' and/or inhibitors' affinities of trout Abcg2a versus human ABCG2 expressed in Sf9 insect cells have been compared as well.

2. Materials and methods

2.1. Chemicals

Triton X-100, Dimethyl Sulphoxide (DMSO), phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS), pyruvate, HEPES, and all compounds tested for their interaction with trout and human Abcg2/ABCG2 (shown on Fig. 7) were purchased from Sigma, St. Louis, MO, USA. The exceptions were MK571 (obtained from Cayman Chemicals Co., Michigan, OR, USA) and calcein-AM (Ca-AM; purchased from Molecular Probes, Eugene, OR, USA). Dulbecco's modified Eagle medium with F12 nutrient mixture (DMEM/F12), Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Karlsrhue, Germany). Ethanol, isopropanol and all other chemicals used were of the highest analytical grade available and purchased from Kemika, Zagreb, Croatia.

2.2. Generation of recombinant plasmids

Designation of gene and protein names used throughout the text is based on the Zebrafish Nomenclature Guidelines:
(https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines); e.g., fish: *shh*/Shh, human: *SHH*/SHH. After sequence verification, trout *abcg2a* was subcloned from pGEM T-easy vector into baculovirus transfer vector pAcHLT (BD Biosciences, Heidelberg, Germany) using *Eco*RI/*Not*I restriction digestion. Subsequently, the same *abcg2a* clone was subcloned into pcDNA3.1-His(+) vector for transfection of human embryonic kidney cell line (HEK293T) using the same restriction enzymes, and to pEGFP vector using EcoRI/*Apa*I digestion to produce recombinant Abcg2a protein with GFP tag fused to the N-terminus. Human *ABCG2* was kindly provided by Dr. Özvegy (Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, Budapest, Hungary) in pcDNA3.1(−) vector and was subcloned into pAcHLT and pcDNA3.1-His (+) vector using NotI/*Kpn*I and EcoRI/*Eco*RV restriction enzymes, respectively.

2.3. Cell culture and transfection

Sf9 insect cells were cultured in TNM-FH medium with 10% FBS. Using the lipofectin reagent cells were co-transfected with recombinant pAcHLT vector containing human *ABCG2* or trout *abcg2a* and baculoviral DNA (BD Biosciences). First baculoviral stock was amplified in two rounds and final baculoviral titer was determined by endpoint dilution assay. Final transfection was made with 2, 4 or 8 multiplicity of infection (MOI) units and after 3 days the cells were collected for membrane preparation.

HEK293T cells were cultured in DMEM high glucose medium with 10% FBS. Cells were transiently transfected with recombinant pcDNA3.1-His(+) plasmids carrying human *ABCG2* or trout *abcg2a*. Polyethylenimine (PEI) was used as transfection reagent in 1:1 ratio to plasmid DNA. After 48 h cells were collected for membrane preparation.

2.4. Membrane vesicles preparation, Western blotting and fluorescence detection

Plasma membrane vesicles were prepared as described by Cornwell et al. (1986). The cells were scraped, washed once in PBS, resuspended and homogenized in TEMP buffer (50 mM Tris, 50 mM manitol, 2 mM EGTA, 2 mM β-mercaptoethanol, 10 μg/ml leupeptin, 8 μg/ml aprotinin, 0.5 mM PMSF). The cell lysate was centrifuged (1000 ×g, 10 min) and layered on 35% sucrose. After centrifugation at 16,000 ×*g* for 30 min in swinging rotor, the layer formed at the top of 35% sucrose was collected, diluted in sample buffer (10 mM Tris, 250 mM sucrose, pH 7.5) and centrifuged at 100,000 ×*g* for 2 h. The pellet containing plasma membrane vesicles was resuspended in 0.5 ml of sample buffer and homogenized by passing the suspension 10 times through a 27-gauge needle with a syringe, and then incubated on ice for 30 min to obtain membrane vesicles. Total proteins were determined by the Lowry method (Lowry et al., 1951). Twenty micrograms of protein per lane were separated by electrophoresis in 7.5% sodium dodecyl sulfate polyacrylamide gel. The proteins were then transferred to polyvinylidene difluoride membrane by semidry blotting. After blocking and washing steps the membranes were incubated overnight with the anti-polyhistidin antibody (Sigma-Aldrich, Taufkirchen, Germany). Goat anti-mouse IgG-HRP was used as secondary antibody (Bio-Rad Laboratories, Hercules, CA, USA). The proteins were visualized using Opti-4CN Substrate Kit (Bio-Rad).

For fluorescence detection, HEK293T cells were grown on glass coverslips in 24-well culture plates. Twenty four hours after transfection with pEGFP vector carrying *ABCG2* or *abcg2a* clone, cells were mounted with Fluoromount, (Sigma-Aldrich, Taufkirchen, Germany) and GFP fluorescence was detected using confocal microscope Leica TCS SP2 AOBS (Leica Microsystems, Wetzlar, Germany).