Human Liver ABC-Transporters – Recombinant Expression and Interactome Studies

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„Zwei Dinge sind zu unserer Arbeit nötig:

Unermüdliche Ausdauer und die Bereitschaft,

etwas, in das man viel Zeit und Arbeit gesteckt hat,

wieder wegzuwerfen.“

Albert Einstein

CONTENTS

I. SUMMARY

ATP-binding cassette (ABC)-transporters are indispensable for the secretion functions of the human liver. Particularly the ABC-transporters of the apical or canalicular membrane of hepatocytes are involved in essential processes such as detoxification and the secretion of bile.

The multidrug transporter ABCG2 and the bile salt export pump (BSEP/*ABCB11*) are in the focus of this thesis. The former participates in the multidrug resistance of cancer cells against chemotherapeutics and the pathogenesis of conditions like gout, while BSEP is the driving force of the enterohepatic circulation of bile salts. Dysfunction of BSEP leads to cholestasis, which causes a number of complications from malabsorption of lipids and fat-soluble vitamins up to liver failure. Despite various *in vivo* studies on these transporters, many aspects, especially molecular mechanisms and their effects on pathogenesis, are not satisfactorily understood.

The present thesis addresses the production of recombinant, purified transporters for *in vitro* investigation particularly in relation to regulative protein-protein interaction.

For ABCG2, expression was established in the fast growing and inexpensive model organism *E: coli*. In the following, the multidrug transporter was purified to more than 85 % homogeneity. Correct folding of the transporter was assessed by its ATPase activity in detergent solution, which was achieved by the addition of the cholesterol analogue cholesteryl hemisuccinate. This established a simple and fast procedure to obtain active ABCG2 for molecular investigation of the transporter such as structure determination.

A previously established overexpression protocol for BSEP in the yeast *Pichia pastoris* was optimized. The purified transporter was used in interaction studies with possible regulatory and stabilizing proteins. Additionally, liver proteins derived from a cDNA library were screened for interaction with BSEP in a yeast two-hybrid approach. A number of ER proteins with functions in topogenesis, quality control and intracellular trafficking were identified. Furthermore, two enzymes involved in bile salt synthesis and the membrane-cortex cross-linking protein radixin were found to interact with BSEP. In addition to the few previously known interaction partners during canalicular protein cycling, these proteins outline a path of interactions for

BSEP from synthesis to the plasma membrane. The eleven identified interaction partners form the basis for further investigations of BSEP trafficking defects, which are a major cause for BSEP-associated cholestasis.

II. ZUSAMMENFASSUNG

ABC (‚ATP binding cassette')-Transporter nehmen bei den Sekretionsfunktionen der menschlichen Leber eine zentrale Rolle ein. Insbesondere die ABC-Transporter der apikalen oder kanalikulären Membran der Hepatozyten sind unabdingbar für Prozesse wie die Entgiftung und Bildung der Gallenflüssigkeit.

Im Fokus dieser Arbeit stehen der Multidrogen Transporter ABCG2 und die Gallensalz Exportpumpe (BSEP/*ABCB11*). Ersterer ist sowohl an der Multiresistenz von Krebszellen gegenüber Chemotherapeutika als auch an der Pathogenese von Krankheiten wie Gicht beteiligt. BSEP ist die treibende Kraft für die enterohepatische Zirkulation von Gallensalzen und Fehlfunktion des Transporters führen zu Cholestase, deren Folgen von Maldigestion insbesondere von Fetten und fettlöslichen Vitaminen bis zu Leberversagen reichen. Trotz weitreichender Untersuchung der Transporter in lebenden Systemen, sind insbesondere die molekularen Mechanismen, welche ein umfassendes Verständnis der ABC-Transporter und der mit ihnen zusammenhängenden Krankheiten ermöglichen, noch nicht ausreichend verstanden.

Die vorliegende Arbeit befasst sich mit der Etablierung und Optimierung von Methoden zur rekombinanten Expression und Reinigung von Transportern für deren *in vitro* Untersuchung. Hier lag der besondere Fokus auf der Aufklärung von regulativen Protein-Protein Interaktionen.

Für den Drogentransporter ABCG2 wurde die Expression in dem schnell wachsenden und günstigen Modellorganismus *E. coli* etabliert. Der Transporter konnte im Folgenden in einem Schritt auf über 85 % Homogenität gereinigt werden. Die korrekte Faltung von ABCG2 in Detergenzlösung konnte über die ATPase Aktivität nachgewiesen werden, die durch das Cholesterin-Analog Cholesterylhemisuccinat erreicht wurde. Somit ist nun ein einfaches und schnelles System vorhanden, um aktives ABCG2 für molekulare Untersuchungen, wie zum Beispiel die Strukturaufklärung zu erhalten.

Für BSEP wurde ein bereits etabliertes Expressionsprotokoll in *Pichia pastoris* optimiert und der gereinigte Transporter in Untersuchungen zu seiner Interaktion mit möglichen regulatorischen und stabilisierenden Proteinen eingesetzt. Zusätzlich wurden Leberproteine ausgehend von einer cDNA Bibliothek mit einem Hefe-Zwei-

Hybrid System auf Interaktionspartner von BSEP untersucht. Es wurden eine Reihe von ER Proteinen mit Funktionen bei der Proteintopogenese, Qualitätskontrolle und im intrazellulären Proteintransport identifiziert. Außerdem wurden zwei Enzyme, die an der Synthese von Gallensalzen beteiligt sind, sowie Radixin, ein Verbindungsprotein von Membran und Zytoskelett, gefunden. Die Identifikation dieser Proteine zeichnet, zusätzlich zu den wenigen, bereits bekannten Interaktionspartnern beim kanalikulären Austausch, den Weg von BSEP von der Synthese bis zur Plasmamembran nach. Es konnten elf bisher unbekannte Interaktionspartner identifiziert werden, welche die Grundlage für weitere Untersuchungen in Bezug auf fehlerhaften Proteintransport bilden, welcher eine häufige Ursache von BSEP assoziierter Cholestase ist.

1. INTRODUCTION

The liver is the largest gland of the human body and performs a number of vital functions. Production of blood plasma proteins and bile, glucose homeostasis, blood filtration, detoxification and iron storage are just a few of the liver's manifold tasks.

Hepatocytes constitute the majority of liver cells and perform many of the liver's essential functions, such as bile production and detoxification. Like all cells, hepatocytes are separated and protected from the external milieu by a lipid bilayer. Since membranes are not permeable for most biologically relevant molecules the cell relies on channels and transporters to facilitate the selective and regulated exchange of molecules with the exterior (1). The liver's many secretory functions depend on active transporters in the membranes to catalyze the uptake or secretion of molecules such as bile salts, nutrients or drugs.

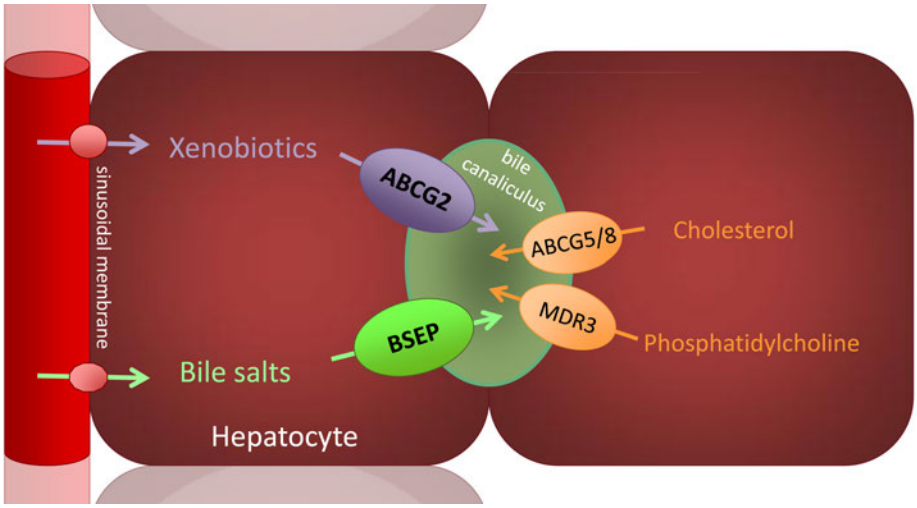

Figure 1 Schematic illustration of canalicular ABC-transporters in the hepatocyte.

At the sinusoidal (basolateral) membrane compounds, such as xenobiotics or circulated bile salts, are taken up from the blood by transporters. After traversing the hepatocyte and possible enzymatic modification, the compounds are secreted into the bile canaliculus mostly by dedicated ABC-transporters, such as ABCG2 and BSEP. Additional bile constituents are cholesterol and phosphatidylcholine, which are translocated by ABCG5/8 and MDR3, respectively.

Hepatocytes are polarized epithelial cells with a set of dedicated transporters (2, 3). Exchange with the sinusoidal blood takes place at the basal membrane while the apical membrane lines the bile canaliculus, where the bile is initially formed (Figure 1) (4, 5).

ATP-binding cassette (ABC)-transporters are a large, ubiquitous family of primary active transporters. The human genome codes for 49 ABC-proteins of which at least nine perform essential functions in the liver. At the canalicular membrane most transporters are ABC-transporters (6), which are responsible for the translocation of a variety of substrates into the bile canaliculus. Examples are the cholesterol transporter ABCG5/8, the lipid floppase MDR3 (ABCB4), the bile salt export pump (BSEP/ABCB11) and the multidrug transporter ABCG2 (Figure 1). Dysfunction of hepatobiliary ABC-transporters causes or aggravates a number of severe diseases, which range from nutrient malabsorption and gallstones to liver cirrhosis and cancer (7). The following paragraphs will introduce the family of ABC-transporters in more detail and focus on this thesis' proteins of interest: ABCG2 and BSEP.

1.1. ABC-Transporter Structure and Function

ABC-transporters constitute one of the largest family of integral membrane proteins. In the current understanding, they function as either importers, which are mainly found in prokaryotic organisms, or exporters, which are ubiquitous in all kingdoms of life. ABC-transporters facilitate the translocation of an extremely wide spectrum of compounds, generally by an ATP-fueled transport process. The transported substrates have been termed allocrites to distinguish them from the substrate ATP (8-10).

The allocrite spectrum of ABC-transporters covers almost every known type of biological compound as well as many synthetic substances. Ions, carbohydrates, lipids, proteins and sterols are just a few examples (11). Many ABC-transporters are specific for one allocrite while others accept a wide range of chemically divers substrates.

ABC-transporters share a distinct domain structure. The nucleotide binding domains (NBD) energize the transport process by binding and hydrolyzing ATP. The energy is transferred as conformational changes to the translocation pathway across the membrane, which is formed by the transmembrane domains (TMD). The functional unit of an ABC-transporter as seen in Figure 2 contains at least two NBDs and two TMDs (10).

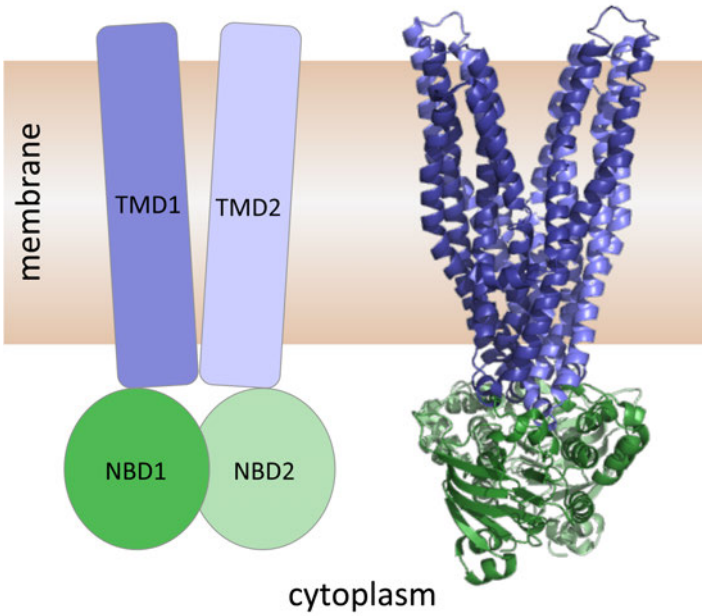

Figure 2 Structure of a bacterial ABC-exporter.

Schema of the functional unit of an ABC-transporter and structure of the bacterial exporter Sav1688 (PDB: 2HYD). Transmembrane domains (TMD) are colored in blue, nucleotide binding domains (NBD) in green.

The NBDs of ABC-transporters show a highly conserved fold and consensus sequences across the species (12). NBDs are L-shaped with a larger core subdomain and an alpha helical subdomain. The core domain contains five conserved motifs, most of which are involved in the binding and hydrolysis of ATP, while the defining signature motif of ABC-transporters is found in the alpha helical subdomain.

TMDs are usually made up of six to ten transmembrane helices and display a low homology across the ABC-transporter family. Consequently, the TMDs are thought to define allocrite specificity (10). ABC-exporters such as ABCG2 and BSEP have six transmembrane helices per TMD.

1.2. Life Cycle and Regulation of Liver ABC-Transporters

Membrane proteins are generally translated at the endoplasmic reticulum (ER) and inserted into the membrane in a co-translational process (13, 14). Recognition of hydrophobic signal sequences by the signal recognition particle leads to association with the Sec translocon, which facilitates the insertion of transmembrane segments into the membrane (15). Depending on the cargo, accessory proteins may be required for this process (16, 17). After the transporter has been inserted into the membrane, it is trafficked to its destination and can undergo posttranslational modification in the secretory pathway.

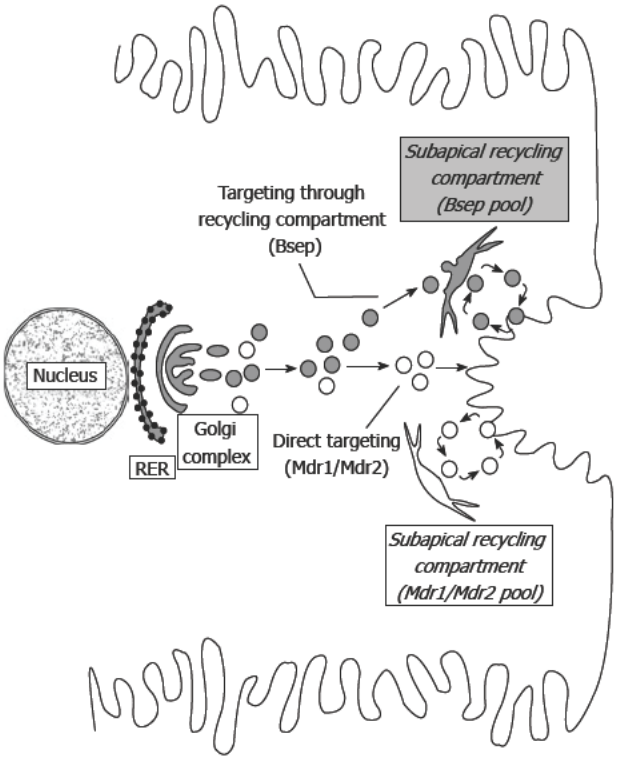

Figure 3 Illustration of routes for trafficking of canalicular ABC-transporters.

The trafficking of vesicles delivering BSEP (gray vesicles) or Mdr1/Mdr2 (white vesicles) from the site of synthesis to the canalicular domain BSEP is indirectly targeted via a subapical, endosomal compartment, which allows the recycling of transporters (exocytic insertion/endocytic internalization). Modified from Roma et al. (18).

The trafficking of membrane proteins is a complex process involving vesicle formation and quality control steps with numerous protein contacts. Membrane proteins traverse the secretory pathway by vesicular transport, where several types of

posttranslational modifications (PTM) are installed along the ER-Golgi pathway. Proteolytic cleavage, formation of disulfide bonds and glycosylation are common modifications (19). Correct PTM and folding are often critical checkpoints, which determine if a transporter reaches the plasma membrane or if is removed from the secretory pathway and degraded (20).

Mature transporters reach the plasma membrane and are inserted by exocytosis. At the canalicular membrane it was observed that some ABC-transporters can undergo multiple insertion and retrieval cycles (21). The proteins can be stored in subapical vesicles and fuse with the cell membrane on demand. At the end of their life cycle, e.g. BSEP has a half-life of about 4-6 days in rats (22), transporters become ubiquitinated and are, after endocytic internalization, degraded in the lysosomal compartment (18).

Expression and function of ABC-transporters is often tightly regulated. Transcriptional and translational regulation controls the long-term availability of a transporter, while post-translational mechanisms influence aspects of trafficking and activity on a shorter time scale (23). Several pathophysiological mechanisms include failure of transporters to target to the plasma membrane, for example in cholestasis (discussed in Chapter 1.4), cystic fibrosis or Tangier disease (24-26).

1.3. The Multidrug Transporter ABCG2

ABCG2 was discovered as an ABC-protein highly expressed in the placenta and at the same time by two other groups through its ability to transport chemotherapeutic drugs (27-29). ABCG2 is widely expressed, particularly in barrier-forming tissue such as the intestinal, placental, renal and liver epithelium and the endothelial blood-brain barrier. Further investigation showed that ABCG2 is a multidrug pump capable of extruding many chemically unrelated compounds (Figure 4) such as chemotherapeutics, antivirals, antibiotics and fluorescent dyes (30). Due to its broad substrate spectrum ABCG2 is attributed with a tissue protective role and belongs to the group of proteins conveying multidrug resistance (MDR) (31). On the other hand, ABCG2 is also capable of transporting a number of endogenous substrates such as sulfated and glucuronidated sterols, urate, folic acid, glutathione, riboflavin and vitamin K_3 (32). The transporter may participate in the cellular homeostasis of these compounds (33).

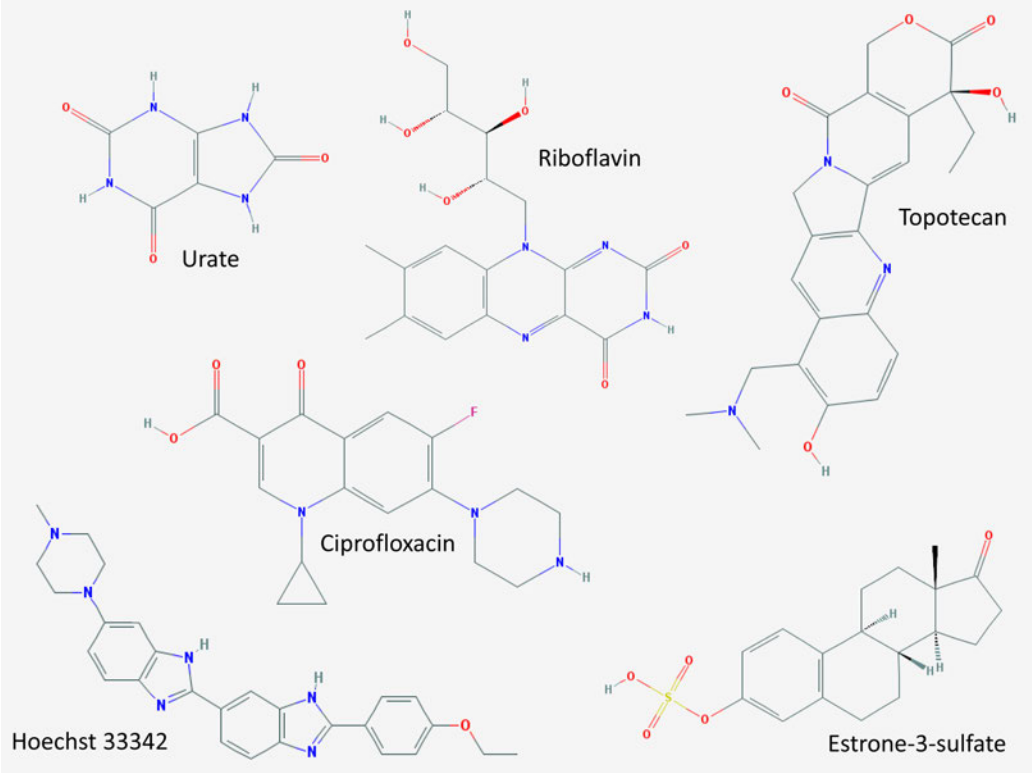

Figure 4 Chemical structure of selected ABCG2 allocrites.

Illustrating the structural diversity of ABCG2 allocrites with the example of three endogenous substrates (urate, riboflavin and estrone-3-sulfate) and three xenobiotics (topotecan, hoechst 33342 and ciprofloxacin).

ABCG2 is a half-size transporter and consequently requires at least dimerization to form a functional transporter, although there are indications that ABCG2 also forms higher-order oligomers (34-36). The *ABCG2* gene codes for a polypeptide with a so-called inverse domain organization, where the NBD is followed by a TMD. The fully modified transporter has a molecular weight of about 72 kDa. Posttranslational modifications include an inter- and an intramolecular disulfide bond, and N-linked glycosylation of residue Asn596 (37). The intramolecular disulfide bond as well as the glycosylation have been shown to influence ABCG2 stability (38-40).

From a medical perspective the impact which ABCG2 and other multidrug exporters like P-gp (ABCB1) and MRP1 (ABCC1) have on the resistance of tumor cells against chemotherapeutics is of great concern (41). While several studies give contradicting results on the role of ABCG2 in MDR of cancer cells the transporter is known to contribute to the distribution and excretion of several studied drugs (30, 32). ABCG2 is also implicated in other aspects of pathophysiology such as gout, which is caused by deposition of urate crystals around the joints and in the kidney. In mice, absence of the high-capacity urate transporter ABCG2 has been connected to higher serum urate levels which promotes the development of gout (42). Another example is ABCG2's apparent role in Alzheimer's disease, as it has been shown to transport amyloid beta peptide (43, 44). ABCG2 is a factor in several other physiological and pathophysiological processes. Since it contributes to the pathophysiology of cancer, gout and several other conditions, ABCG2 is of great interest in terms of structure and molecular function.

1.4. The Bile Salt Export Pump

Bile salts are amphipathic molecules synthesized from cholesterol in the liver and conjugated to taurine or glycine (45). In the so-called enterohepatic circulation bile salts are secreted from the liver into the intestine, where more than 90 % are reabsorbed and return to the liver via the portal blood (Figure 5). In the intestine the detergent properties of bile salts enable the efficient absorption of lipids and fat-soluble vitamins. Additionally, through bile salt receptors in various human tissues, bile salts act as signal molecules with systemic effects (46-48). Synthesis and distribution of bile salts are tightly controlled because of their toxic detergent effect on surrounding hepatocellular membranes (49). In bile, their toxicity is reduced by integrating bile salts into mixed micelles consisting mainly of phosphatidylcholine and cholesterol (5).

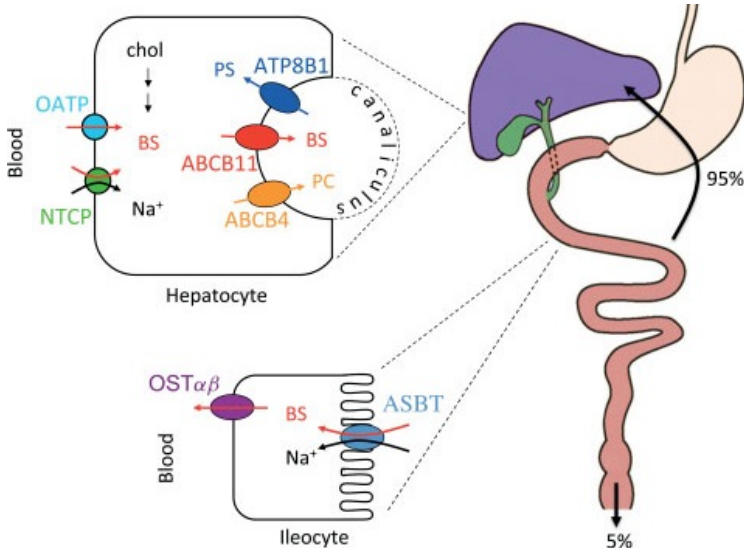

Figure 5 The enterohepatic recycling of bile salts (BSs).

Approximately 95% of the secreted BSs are re-absorbed in the enterohepatic cycle. De novo synthesis from cholesterol (chol) in the liver replaces the 5% that is lost in the feces. Newly synthesized BSs are effluxed into the bile canaliculus by the bile salt export pump ABCB11, where they form micelles with phosphatidylcholine (PC). In this particular scheme cholesterol is not included as a bile component. In the distal ileum, the apical sodium-dependent bile salt transporter (ASBT) imports BSs into the ileocyte. At the basolateral membrane of the ileocyte, BSs are exported by the heterodimeric organic solute transporter OSTα/β into the portal vein and from there to the liver. At the basolateral membrane of the hepatocyte, BSs are imported mainly via the Na+-dependent taurocholate co-transporting polypeptide (NTCP). Members of the organic anion transporting polypeptides (OATPs) also import BSs. Modified from Nicolaou et al. (7).

After synthesis or reuptake in the hepatocytes, bile salts are secreted into the bile ducts in conjunction with other bile components and become up to a thousand times more concentrated. This steep gradient is established by the bile salt export pump (BSEP, *ABCB11*). BSEP is the primary bile salt transporter at the apical membrane and is considered to be the driving force of bile salt dependent bile flow (5). BSEP preferentially transports monovalent bile salts, for example the primary bile salts taurocholate and taurochenodeoxycholate, while unconjugated bile acids such as cholic acid are not transported (50, 51)

BSEP is a full-size transporter with a molecular weight of about 160 kDa. As a glycoprotein BSEP requires a certain degree of glycosylation for proper protein stability and trafficking (52, 53) although it is currently not known which glycosylation sites are essential in human BSEP. Phosphorylation also poses a possible regulative step in BSEP trafficking (54), while ubiquitination has been shown to shorten BSEP half-life at the membrane (55).

BSEP expression, trafficking and function are closely regulated. Transcriptional regulation hinges on the nuclear farnesoid X receptor (FXR), which is a cellular bile salt sensor (56). On a posttranslational level various signals modulate BSEP localization and activity. For example, hypoosmolarity (57), cAMP (22), ursodeoxycholate (58) or phosphorylation (54) lead to BSEP recruitment to the membrane, although the exact molecular mechanisms for these regulative steps are largely unknown.

BSEP is essential for bile flow; consequently functional impairment of the transporter has severe consequences in the form of cholestasis. Patients initially have symptoms such as pruritus and jaundice. They face malabsorption of lipids and fat-soluble vitamins and in later stages liver fibrosis, cirrhosis and liver failure (59). Temporary stimuli in pregnancy or by drugs can cause intrahepatic cholestasis of pregnancy or drug-induced cholestasis, respectively, while hereditary dysfunction of BSEP leads to benign recurrent intrahepatic cholestasis type 2 (BRIC2) or progressive familial intrahepatic cholestasis type 2 (PFIC2) (59-61). Due to its pivotal role in bile secretion, an understanding of the molecular function of BSEP and its regulation are of major importance.

1.5. Aims

The two ABC-transporters ABCG2 and BSEP provide essential functions in the human liver during detoxification and bile production. Although there is a large body of clinical and *in vivo* studies available in regard to molecular function and regulation of these transporters there is still much information lacking, such as the posttranslational regulation by protein-protein interaction (PPI) and the high-resolution structure.

Molecular investigations are an indispensable tool for gaining detailed information on aspects like structure, kinetic parameters and interactions of a protein. Combined with *in vivo* data, which provide the complex physiological background, *in vitro* investigation contributes an important puzzle piece for a full understanding of the molecular mechanisms underlying ABC-transporter function and regulation.

1.5.1. Recombinant Expression of ABCG2

The multidrug transporter ABCG2 is a contributor to cancer cell MDR. Furthermore, by transporting endogenous substrates ABCG2 also participates in the pathogenesis of conditions such as gout and Alzheimer's disease. To investigate a transporter *in vitro* a common prerequisite is a recombinant source to obtain the required amounts of pure protein.

One aim of this thesis is to establish a fast, high-yield expression system for active ABCG2 in *E. coli*.

1.5.2. Interactome of BSEP

BSEP provides the driving force for the enterohepatic circulation of bile salts. Dysfunction of this essential ABC-transporter leads to cholestasis with further complications such as cirrhosis and cancer. One important step to ensure canalicular BSEP expression is the trafficking to and the cycling at the membrane. Several mutations are known to cause trafficking defects, but knowledge of the molecular mechanisms and regulation, in which PPI takes a key role, is limited.

A full understanding of the posttranslational regulation of transporters by PPI requires complementary approaches. These need to cover the physiological situation as well as

the sensitive, high-throughput methods which are currently limited to a non-human context and involve genetic approaches like the yeast two-hybrid or co-purification assays such as tandem affinity purification or *in vitro* pull-down analyses (62).

The second aim of this thesis is to screen for and investigate unknown protein interaction partners of BSEP with a possible regulative role by complementary approaches.

Overall, the aim of this thesis is to expand and utilize the potential of high-yield, recombinant production of human liver ABC-transporters and to explore their interactome towards regulative PPI. Investigation of BSEP and ABCG2 will advance our knowledge in the field of ABC-transporters and add the basis for a more detailed examination of the complex mechanisms that involve their regulation.

2. PUBLICATIONS AND RESULTS

2.1. Chapter 1 – Hepatobiliary Transport in Health and Disease: Structure and Function of Hepatic ABC-Transporters

Contribution to this publication: 30 %

- Writing of the manuscript

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Impact Factor: not available

2 Structure and function of hepatic ABC transporters

Philipp Ellinger, Marianne Kluth, Susanne Przybylla, Sander H. J. Smits, and Lutz Schmitt

2.1 Introduction to human ABC transporters expressed in the liver

Several membrane transporters that belong to a group of ATP-dependent primary transporters, the so-called ABC (ATP binding cassette) transporters, are found in the human genome. In general, ABC transporters contain two transmembrane-spanning domains (TMDs) and two characteristic nucleotide-binding domains (NBDs) localized in the cytosol. In the membrane the two TMDs form a pore-like structure, which facilitates substrate transport against a chemical gradient. One TMD is predicted to have six α-helices, whereas the soluble NBDs are essential for the supply of energy by hydrolysis of ATP. Compared with the TMD, the NBD harbors highly conserved sequence motifs: the Walker A (GXXGXGKS/T, where X can be for any amino acid), Walker B (ΦΦΦΦD, where Φ can be any hydrophobic residue) motifs, and the C-loop (ABC–signature motif, LSGGQ) (1). The C loop, which is located roughly 90 amino acids downstream of the Walker A motif and roughly 30 amino acids upstream of the Walker B motif, is actually the characteristic sequence motif of this family; together with the Walker A and B motifs, it serves as a diagnostic clue to the identification of new family members. Additional sequence motifs present in ABC transporters are the Q loop, the D loop (SALD), and a highly conserved histidine residue essential for ATP hydrolysis, which is positioned 30 amino acid downstream of the D loop (2).

To achieve a thermodynamic uphill transport of the substrate, transport has to be coupled to the cycle of ATP hydrolysis. Several high-resolution structures of full-length ABC transporters and isolated NBDs, in combination with biochemical analysis, have provided important contributions to a molecular understanding of substrate binding, ATP hydrolysis, and substrate transport. For example, the highly conserved NBD has an L-shaped structure consisting of a catalytic domain and a helical domain. The catalytic domain contains the Walker A and B motifs while the helical domain harbors the C loop. These two domains are connected by the Q and Pro loops (3). Further analysis of, for example, the isolated haemolysin B-NBD demonstrated that in the presence of ATP, the two NBDs form a homodimer (2). The Walker A and B motifs of one NBD and the C loop of the opposing NBD bind one ATP, so that the two NBDs are set in a head-to-tail arrangement. This ATP-induced dimerization generates mechanical work, which in principle can be transmitted to the TMDs and might serve as another source of energy (see section 2.2.4). The dimeric NBDs cooperate in hydrolysing ATP and provide the free energy to drive the directional transport of the substrate against a concentration gradient. After ATP hydrolysis, ADP and P_i dissociate from the NBD, the dimer falls apart, and the ground state of the NBDs is restored.

Furthermore, different models for the transport mechanism have been proposed. The simplest model, the alternating access model, describes two basic conformations. One conformation is open to the cytosolic side (inward-facing), with a substrate-binding

site accessible for the substrate only from the cytosol, and the second conformation is open to the extracellular side, containing a binding site, which has a low affinity to the substrate and is accessible only from the extracellular space (4). A continuous model is the ATP-switch model. In the ground state the transporter is in the inward-facing conformation with a high-affinity substrate-binding site and the NBDs exist as monomers, with low affinity to ATP. The ATP-induced dimerization of the NBDs leads to a conformational change in the TMDs such that the substrate-binding site is exposed to the extracellular space, the substrate affinity is reduced, and the bound substrate is finally released (5). However, the exact molecular coupling of the ATP–hydrolysis cycle and substrate transport is still not entirely clear.

In the human hepatocyte, several ABC transporter are expressed: for example, the bile salt export pump (BSEP, ABCB11), responsible for bile salt transport; ABCG5/ABCG8, involved in sterol transport; multidrug resistance protein 3 (MDR3, ABCB4), flopping phosphatidylcholine from the inner to the outer membrane leaflet; and ABCG2, transporting a variety of hydrophobic substances (▶Fig. 2.1). Mutations in one of these transporters are associated with different kinds of liver diseases of varying severity. For example, Dubin-Johnson disease is related to mutations in MRP2 (ABCC2), and progressive familial intrahepatic cholestasis type 2 (PFIC2) is associated with a mutations with the bile salt export pump BSEP.

This chapter summarizes experimental insights and focuses on the canalicular ABC transporters BSEP, MDR3, and ABCG2, highlighting their discovery and evolution and the *in vitro* assays from which a mechanistic understanding may be derived.

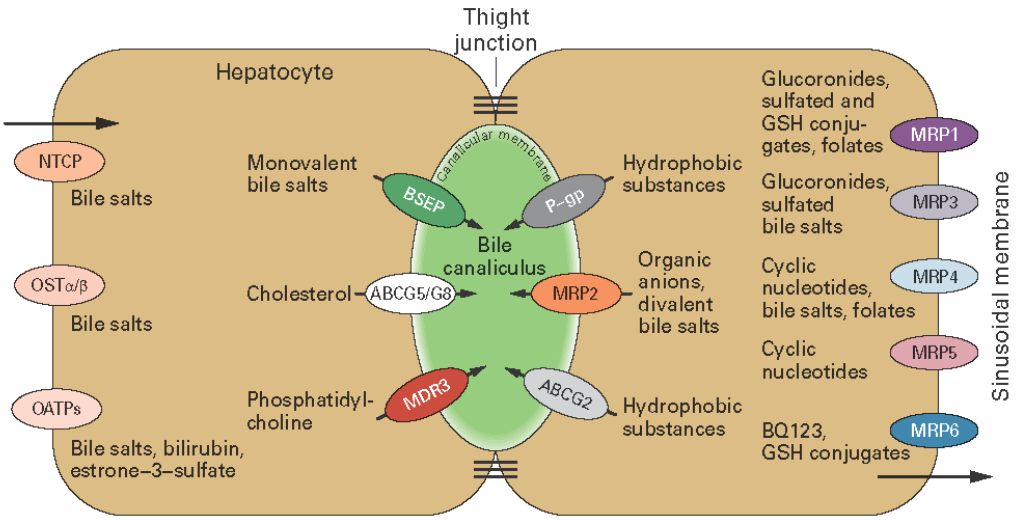

Fig. 2.1: Localization of transporters in the hepatocytes. Bile salts are taken up at the sinusoidal (basolateral) membrane through the sodium–taurocholate cotransporting peptide (NTCP) in a sodium–dependent manner and to a lesser extent through a sodium–independent transport by organic anion transporting proteins (OATPs). They are then further shuttled to the canalicular membrane and transported via the bile salt export pump (BSEP) into the canaliculus. Multidrug resistance protein 3 (MDR3) and Sterolin 1

(*Continued*)

Fig. 2.1: (*Continued*)

(ABCG5/G8) complete the bile formation by flopping phosphatidylcholine (PC) from the inner to the outer leaflet as well as transporting cholsterol. Bile salts, PC and cholesterol form mixed micelles which constitute the basis of bile. P–glycoprotein (P–gp) and the breast cancer resistance protein (ABCG2) transport a variety of hydrophobic substances into the bile and confer multidrug resistance (MDR). Furthermore, a substanial number of multidrug–related proteins (MRPs) are localized in the sinusoidal membrane except MRP2. They transport a broad range of organic anions and conjugated substances. They also participate in MDR and some of them, e.g. MRP4 as well as the organic solute transporter (OST) act as salvage system for too high bile salt concentrations within the cell to prevent toxicity.

2.2 Structure and function of the bile salt export pump (ABCB11; BSEP)

2.2.1 Liver transport of bile salts

Bile salts are essential for the absorption of lipids and fat-soluble vitamins, originated from food intake, by the enterocytes of the small intestine and also for the excretion of endo- and xenobiotics with the bile. They are synthesized by multiple enzymatic reactions in the liver, more precisely in the hepatocytes from cholesterol as educt; this constitutes one of the key function of the liver (6). From there bile salts enter the biliary tree and are stored in the gallbladder upon food intake (7). After they have fulfilled "their mode of action", bile salts pass through the enterohepatic circulation, meaning that they are reabsorbed to ~90% in the small intestine and then transported back to the liver via the portal blood. There, they are transported again into the hepatocyte and the cycle starts anew with their secretion into the canaliculi (8,9). A single bile salt molecule traverses the cycle approximately up to 10 times a day until it is excreted via the intestine, which makes this circulation an extremely efficient recycling system (10).

Because bile salts are amphipathic molecules, they display a detergent character. Hence a high concentration within the cell is deleterious, leading to damaged mitochondria and apoptosis or necrosis of the hepatocytes owing to the salts' ability to solubilize or create defects within biological membranes. To prevent this and keep bile salts circulating, a specialized set of bile salt transporters in the hepatocyte is required (9,11,12). In the basolateral membrane (also called the sinusoidal membrane), bile salts are taken up from the portal blood. This is accomplished by the sodium taurocholate cotransporting peptide (NTCP, SLC10A1) in a sodium-dependent transport process (13). In addition, there is the less frequently used sodium-independent transport by the organic anion-transporting polypeptides (OATPs) (14).

After entering the cell, bile salts reach the apical membrane (also called the canalicular membrane); the exact mechanism of this is not yet completely understood. For example, one mechanism involves bile salt–binding proteins (15).

At the canalicular membrane, bile salts are transported into the canalicular lumen by the ATP-binding cassette transporter (ABC transporter) bile salt export pump (ABCB11;

BSEP) (16,17). BSEP is the main driving force for the bile salt–dependent part of bile flow and a bottleneck in the enterohepatic circulation. It must transport bile salts against a steep concentration gradient to maintain circulation, since the concentration of bile salts in the canaliculus is 1000 fold higher than in the cell, 1 mM and 1 µM, respectively (10).

2.2.2 Discovery of the bile salt export pump

The electrochemical gradient across the canalicular membrane is ~ −35 mV and its discovery marked the first explanation for bile salt transport across this membrane (18,19). However, this electrochemical gradient alone could not be the entire explanation. Finally, in 1991, an ATP-dependent system for the transport of taurocholate in isolated canalicular membranes of rat liver was described. Other laboratories subsequently confirmed this finding (20,21). Evidence that an ABC transporter was responsible for bile salt secretion into the canaliculus appeared in 1995. It involved an increased level of mRNAs, detected by Northern blotting, in combination with the overexpression of an ABC transporter found via the Western blot technique with a P-glycoprotein antibody (ABCB1, MDR1, P-gp); these were demonstrated in a bile salt–resistant rat hepatoma–derived cell line (22). This suggested that an ABC transporter closely related to P-gp became upregulated in this system. In the same year, Childs et al. screened a pig cDNA library with a probe consisting of a P-gp sequence and identified a gene exclusively expressed in the liver that had a sequence identity of 61% to human P-gp on the amino acid level (23). This gene was named "sister of P-gp" (sP-gp), but its function remained unknown. Gerloff et al. were the first to demonstrate that oocytes exhibited a stimulated taurocholate efflux when liver sP-gp cRNA was injected into *Xenopus laevis* oocytes and the first to express sP-gp in *Sf*9 (*Spodoptera frugiperda*) cells (24). Furthermore, membrane vesicles derived from these *Sf*9 cells demonstrated an ATP-dependent taurocholate uptake, much as in previous studies with isolated canalicular membranes. Because of these findings the "sister of P-gp" was renamed "bile salt export pump (BSEP)" and was considered to be the predominant bile salt transporter in the apical membranes of hepatocytes (24). Further strong support for this consideration was obtained by positional cloning of the human *BSEP* gene and mapping it to chromosome 2q24, a locus linked to progressive familial intrahepatic cholestasis type 2 (PFIC2), a severe liver disease (25).

2.2.3 Evolution of the bile salt export pump

The production of bile salts and their subsequent transport into the canaliculi is highly conserved among the livers of vertebrates. Over the years, BSEP has been detected and studied in the pig (23), rat (26), mouse (27,28), rabbit (29), dog (30) and human (31,32). Interestingly, full-length BSEP cDNAs has been identified in a variant of the small skate (*Raja erinacea*), a 200-million-year-old marine vertebrate with an amino acid sequence identity of 68.5% to the human orthologue (33). Here, bile salts are transported in large amounts by BSEP. Furthermore, it was demonstrated that mutations leading to PFIC2 in humans had the same effect on substrate transport in skate BSEP (33,34). The bile of this elasmobranch normally consists of bile alcohols (scymnol sulfate) rather than bile salts, which cannot be found in its bile. Probably bile alcohols were the original

substrates for BSEP, and mammalian evolution led to different substrates owing to a selective pressure – for example, more fat in the diet. Interestingly, skate bile does not contain any phospholipids and no MDR3 protein (a phospholipid floppase) is found in the hepatocyte. The function of BSEP and its tight correlation with MDR3 is described in section 2.3. Importantly, however, this finding suggests that BSEP evolved much earlier than the highly identical MDR3 protein (sequence identity between P-gp and MDR3 of ~80%) and probably also by gene duplication (35). The occurrence of lipids in bile was potentially the result of the more deleterious bile acids than of the bile alcohols that arose during evolution. All of these indications demonstrate that BSEP diverged very early from P-gp and that it is highly conserved in vertebrate evolution.

2.2.4 The bile salt export pump – a member of the ABC transporter family

BSEP belongs to the group of ABC transporters. They can be found in all the taxonomic kingdoms (from bacteria to humans), and all possess the same modular architecture and act either as importers or exporters (36). In humans, 48 ABC transporter genes have been identified in addition to a small number of pseudogenes, which are not expressed (37). All known eukaryotic ABC transporters are exporters, whereas ABC importers can be found only in Archaea and Bacteria. In humans, ABC transporters are expressed throughout the body, but some highly tissue-specific and ABC transporters are restricted to the liver (37). Phylogenetic analysis of the entire human ABC transporter sequences has led to the classification of seven subfamilies (A to G) (38). Because of their important roles in human physiology, dysfunction is the cause of very severe diseases, such as cystic fibrosis (39). In terms of mutations of liver ABC transporters BSEP and MDR3, for example, PFIC2 (25) and PFIC3 (40) may develop (see chapter 9). BSEP belongs to the group B (MDR/TAP) subfamily of human ABC transporters because of its high sequence identity to P-gp. The gene is located on chromosomes 2q24 (25) and the 28 exons code for a 1321 amino acid glycosylated ABC transporter with a molecular mass of ~160 kDa (31,32).

ABC transporters have a core architecture consisting of two NBDs and two TMDs. In eukaryotes these modules are encoded on a single gene, but one must distinguish between the full-size transporters (two TMDs and two NBDs) and half-size transporters (only one of each domain). The latter homo- or heterodimerize to form a functional transporter. BSEP is a full-size ABC transporter with a core molecular weight of 146 kDa. Interestingly, the N-terminal NBD (NBD1) of BSEP contains a methionine instead of a glutamate within the Walker B motif. The glutamate normally interacts with ATP through a catalytic water molecule that catalyzes the nucleophilic attack onto the γ–phosphate. ATP binding sites in ABC transporters are composed of the Walker A and B motifs of one NBD and the C-loop of the other NBD. Therefore the ATP-binding site that contains NBD1 (site 1) is a degenerated site. Degeneration of this conserved residue is also seen in other human ABC transporters like TAP1/2 or CFTR (41). Functional studies of other degenerated ABC transporters have demonstrated that this mutation leads to an ATP-deficient site within the NBD dimer. This, of course, implies an asymmetric function of the two NBDs and further suggests that ATP binding site 1 of BSEP is catalytically inactive or active only at drastically reduced levels compared with the other ATP binding site in the composite dimer. This phenomenon has not been investigated for BSEP so far but is of high concern for a molecular understanding of ATP hydrolysis coupled to bile salt transport. The TMDs are located within the membrane and provide the translocation

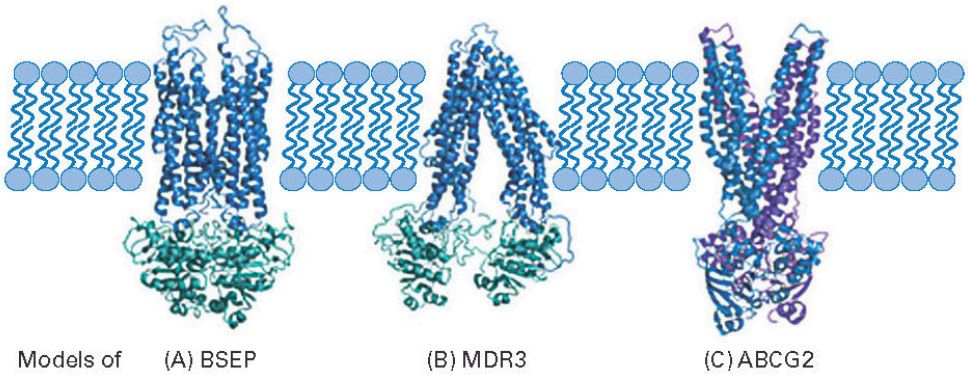

Fig. 2.2: Models of BSEP, MDR3 and ABCG2. (**A**) The model of BSEP based on the known structure of Sav1866 from *Staphylococcus aureus*. The transmembrane domain is highlighted in blue and the nucleotide binding domain in cyan. The used template is deposited under protein data bank (PDB) code 2HYD (**B**) The model of MDR3 based on the known structure of P-gp from *Mus musculus*. The transmembrane domain is highlighted in blue and the nucleotide binding domain in cyan. The used template is deposited under PDB code 3G61. (**C**) The model of ABCG2 based on the known Structure of the multidrug ABC transporter Sav1866 from *Staphylococcus aureus* in complex with AMP-PNP. The used template is deposited under PDB code 2ONJ. Since ABCG2 is a halfsize transporter the two monomers are colour coded differently. Monomer I is highlighted in blue and monomer II in purple. Due to the bound AMP-PNP in the template structure the conformation of ABCG2 represents the potential nucleotide bound state. It is important to clarify that these models are based on the known X-ray structure and the structures obtained from the actual protein might look differently.

pathway for the substrate. In contrast to the NBDs, the TMDs are highly variable in their sequence and thus determine the substrate specificity. It is assumed that many human ABC transporters show the 6 × 6 topology, meaning that they contain six TM helices (TMH) traversing the membrane followed by a cytoplasmically located NBD and again six TMHs and an NBD. This assumption was originally proposed based on cysteine scanning mutagenesis of P-gp and the recently developed x-ray structures of mouse P-gp and bacterial homologues (42); it may be true for BSEP as well. BSEP is also a full-size ABC transporter containing 12 TMHs and two NBDs (▶Fig. 2.2). This number of helices is derived from hydrophobicity calculations because structural information on BSEP is lacking. To date only one eukaryotic ABC transporter structure (of mouse P-gp) has been published (42). The second available structure (ABCB10) is deposited only in the Protein Data Bank (PDB database). The P-gp structure shows the typical bundle of six helices crossing the membrane. However, as first observed for Sav1866, a domain swap is present in P-gp, suggesting that such a swapping is a conserved feature of ABC drug pumps. Here, four helices of one bundle and two helices of the other bundle build up one TMD. The TMDs provide a large cavity for substrate binding for mouse P-gp, which may also be true for BSEP. According to the "cholesterol fill-in mechanism," cholesterol also participates in substrate recognition and fills the volume of the cavity that is not occupied by the substrate, as postulated for P-gp. Besides the amino acids, which constitute the substrate-binding site, this could also e an additional explanation for the fact that those two closely homologous transporters have different substrate spectra and

BSEP is restricted to bile salts. It has been demonstrated that the activity of BSEP critically depends on cholesterol. This might be because BSEP is targeted to detergent-resistant microdomains (DRMs) in the canalicular membrane, which exhibit a high amount of cholesterol and sphingomyelin, or because of the "cholesterol fill-in model." Whatever the molecular reason(s) for the differences between P-gp and BSEP may be, all the models proposed must be verified experimentally for BSEP in the future. So far most if not all functional information on BSEP results from disease-linked mutations found in patients with, for example, PFIC2 (see chapter 9) and offers insights into the way single amino acids influence the trafficking, stability, and transport capabilities of BSEP.

2.2.5 Cloning and expression systems for BSEP

To study a protein biochemically *in vitro,* it is often necessary to obtain sufficient amounts of pure, homogeneous protein. Therefore an expression system must be chosen and recombinant expression constructs must be cloned. All this is true for BSEP, but it has one big drawback. The human cDNA that codes for BSEP has been found to be unstable in *Escherichia coli* (31,32,43). This phenomenon has been observed for several other mammalian membrane proteins as well (44). Because *E. coli* is the most widely used cloning and expression host for standard molecular biology techniques, other strategies must be applied in utilizing the favored expression system. In the case of human BSEP, after several years of struggle, efforts to clone the cDNA into an expression vector were eventually successful. However this led, even after a bacterial promoter in the cDNA was silenced, to a construct with several point mutations within the coding sequence (six missense mutations) and the loss of specific parts of the coding sequence during expression construct propagation (32). All these findings led to the notion that the cDNA of BSEP is "toxic" or "unstable" for cloning and/or the expression host since colonies would no longer grow. One way of circumventing this laborious work is to use homologous recombination (HR) in the yeast *Saccharomyces cerevisiae* (45,46). We therefore established a workflow for human BSEP that can also be applied to any other target (47). Here the expression vector of interest was modified by the introduction of an origin of replication (ori) and a selection marker for *S. cerevisiae* into the backbone of the plasmid. The linearized expression vector and the PCR-amplified BSEP cDNA, which has overlapping ends to the expression vector, are then transformed into yeast. *S. cerevisiae* is capable of recombining those overlapping ends to a circular vector, and only clones that do this correctly are able to grow under selection. The expression vector can be recovered from yeast and transformed in *E. coli* for amplification. We have found that *E. coli* is capable of handling the BSEP cDNA if it is in a closed, circular plasmid form (no nicks, etc.) and grown strictly at or below 30°C. If required, our expression construct can be designed to remove the origin of replication or selection marker to prevent a potential influence of these additional sequences on balanced expression systems. Furthermore, we developed a mutagenesis strategy relying only on yeast. A changed primer design (primers carrying the mutation are not completely complementary to each other) results in a PCR product with overlapping ends (5′ and 3′ ends) that can be recombined by yeast, resulting in a plasmid containing the desired mutation. With the directed recombination–assisted mutagenesis (DREAM) method, mutations can be introduced more easily and quickly than with commercially available strategies. Therefore this method is seen as a DREAM (47).

The major bottleneck in studying membrane proteins in vitro (e.g. structural and functional studies), is their homo– or heterologous overexpression, making it difficult to purify the protein in adequate amounts. Therefore one must choose between prokaryotic and eukaryotic expression systems. As a prokaryotic expression system, *E. coli* is the most widely used host (48), although there are others like *Lactococcus lactis*, which is also successfully used for the overexpression of membrane proteins (49). Mammalian transporter can be expressed in *E. coli*, but sometimes in an inactive manner. Therefore we also tried to overexpress human BSEP in *E. coli* using the T7-RNA polymerase/promotor system in combination with a synthetic gene, which sequence was optimized for use in *E. coli*. Unfortunately cells stopped growing upon induction of BSEP expression and we were not able to detect BSEP in cell lysates via Western blotting. BSEP is a plasma membrane protein and *E. coli* does not possess the eukaryotic posttranslational modification system, and no cholesterol is present in the inner membrane. Therefore, eukaryotic expression systems are likely the methods of choice for BSEP. Three different expression systems, which are also commercially available, are used the most: yeast, insect, and mammalian cell lines. Mammalian cell lines have the great advantage that they present the native environment of BSEP; these cells contain the native lipid environment, the native secretory/posttranslational pathways, and a known functional expression. Human BSEP could be expressed in different mammalian cell lines (e.g. HEK293 cells (50), HepG2 cells (51), MDCK cells (52) and LLC PK1 (53) cells) and characterized functionally without purification. The most widely used system for the heterologous expression of BSEP is the insect cell system. Human BSEP was expressed in *Sf*9 (32,54) as well as HighFive cells (31). Insect cells exhibit a nonnative lipid environment with low levels of cholesterol as well as nonnative glycosylation (generally of the high-mannose type), but they resemble the native conditions more than yeast does. Expression in this system is used to investigate the transport properties of BSEP in vesicular-based transport assays. Cell culture–based systems may generally be suitable for addressing questions of a cellular phenotype, protein trafficking, and the modification of protein interactions. But mammalian and insect cell systems are also costly and maybe not be producible in large the amounts required for purification and structural studies of BSEP. From this point of view, a better choice might be yeast. Two yeast-based systems are used to overexpress mammalian membrane proteins: the previously mentioned *S. cerevisiae* and *Pichia pastoris*. The advantages are obvious: yeast is inexpensive, requires simple culture media, and exhibits well-studied genetics. Furthermore, yeasts have the eukaryotic modification machinery. Of course there are disadvantages, since, for example, the lipid composition of the membrane is different from that of mammalian cells (they contain ergosterol instead of cholesterol) and *S. cerevisiae* often hyperglycosylates proteins (highly branched and extended high-mannose structures), which is not observed in *P. pastoris*. It has been shown that BSEP requires glycosylation for transport activity in MDCK cells (55), although the type of glycosylation seems not to be important, as shown by functional expression in insect cells. *S. cerevisiae* was also used to express human P-gp (56) and MRP1 (57), and we were also able to express BSEP in this host (unpublished data), yielding only low amounts of fully translated protein. Therefore we switched to *P. pastoris*. This yeast was shown to overexpress 25 human ABC transporters (BSEP was not among them) and was also the expression host for mouse P-gp, which in the end and after a long endeavor resulted in the three dimensional x-ray structure (42,58). Recently we demonstrated the heterologous overexpression of human BSEP in

this yeast (47). Another advantage of *P. pastoris* is that this methylotrophic yeast strain can be fermented to high cell densities generating large amounts of biomass, which can be used for subsequent purification.

2.2.6 *In vitro* assays to study BSEP

Although BSEP has not been purified to homogeneity yet, assays have been described to study the function of BSEP *in vivo*. A vesicular transport assay is the most important one. It consists of three steps: (a) preparation of membrane vesicles, (b) addition of substrate and an energy source, and (c) readout of substrate uptake into the vesicles. In general there are two ways to prepare membrane vesicles from cells, right-side-out (RSO) and inside-out (IO) vesicles. In RSO vesicles, the cytosolic side of the transporter is localized in the lumen of the vesicles, whereas in IO vesicles it is vice versa. The latter ones are commonly used for primary transporters such as BSEP. With the addition of ATP and substrate, transport is initiated and the substrate begins to accumulate in the lumen of the vesicles. After a defined amount of time, the reaction is stopped by, for example, a rapid-filtration method; then the transported amount of substrate, which is retained in the vesicle on the filter, is quantified (via radioactivity, fluorescence, or LC/MS). With this assay, the substrate spectrum of BSEP was elucidated (see ▶Tab. 2.1 for human BSEP). These assays were mainly performed with BSEP derived from insect cell vesicles (31,32) but also with vesicles originated from HEK293 cells or isolated canalicular membranes (52). Human BSEP transports monovalent conjugated bile salts in the order of taurochenodeoxycholate > taurocholate > tauroursodeoxycholate > glycocholate (it has to be

Tab. 2.1: Substrate spectra and Michaelis-Menten constant for human BSEP from different expression systems. For an excellent overview, see reference 60.

Substrate	K_M / µM	Source
Taurocholate	8 (32), 20 (30, 61), 15(54)	Sf9
	4 (31)	HighFive
	6 (50)	
Taurochenodeoxycholate	4 (54), 5 (32), 13 (61)	Sf9
	7 (50)	HEK293
Tauroursodeoxycholate	12 (32)	Sf9
Taurodeoxycholate	34 (61)	Sf9
Taurolithocholate	4 (61)	Sf9
Taurolithocholate 3 sulfate	10 (50)	HEK293
Glycocholate	11 (32), 36 (54)	Sf9
	22 (50)	HEK293
Glycochenodeoxycholate	2 (54)	Sf9
	8 (50)	HEK293
Pravastatin	124 (62)	HEK293

mentioned, that the K_M values vary slightly between different expression systems but not unconjugated ones) (see ▶Tab. 2.1). In most of these studies bile acids are tritium-labeled for readout, but fluorescent bile acid derivatives, such as cholylglycylamidofluorescein and chenodeoxycholylglycylamidofluorescein, have also been investigated (59). In addition, inhibitors were analyzed for their potential impact on BSEP. Inhibition of BSEP by different drugs causes drug-induced cholestasis, leading to severe liver injury (63). Examples of inhibitors that were determined for human BSEP by a vesicular uptake assay in competition experiments with bile salts include cyclosporine, rifampicin, and bosentan. These assays and the recommendation of the European Medicines Agency (64) emphasize the importance of BSEP for drug development. With a vesicular uptake assay for BSEP commercially available, the screening of drug libraries is in principle straightforward. One disadvantage of this kind of assay, however, is that besides the target transporter, the vesicles contain many irrelevant membrane proteins that probably affect the uptake assay. This can be excluded if proper controls are performed, but it complicates the assay.

Mutations in the *BSEP* gene can lead to an impairment of bile salt transport due to the protein's dysfunction. This can lead to PFIC2 or BRIC2, a severe liver disease, which at present can be cured only by liver transplantation (65). Currently, according to the Human Gene Mutation Database (http://www.hgmd.org/), 179 disease-related BSEP mutations are known. Thus an understanding of the effect of such mutations could, in the future, lead to therapeutic innovations that might cure this disease without transplantation. If, for example, mutated BSEP is still able to transport and the disease is caused by a trafficking defect, it could also be investigated by the vesicular transport assay, with mutation and localization studies in cell culture systems involving immunostaining or with a fluorescent tag like eGFP or YFP.

Trafficking and the regulation of BSEP in the apical membrane of hepatocytes also requires adaptor proteins. HCLS1-associated protein X-1 (Hax1), for example, was identified using yeast two-hybrid screens as well as pull-down assays with glutathione-S-transferase (GST) tag fusion proteins (soluble parts of BSEP with GST tag) and co-immunoprecipitation (66). Other adaptor proteins are still not known and would be of high interest, especially for the short-term regulation of BSEP or for their potential involvement in trafficking mutants.

2.3 Structure and function of the multidrug resistance protein 3 (ABCB4; MDR3)

As described in section 2.2.1, BSEP is essential for the circulation of bile salts. However, bile salts are harsh detergents and possess the power to solubilize any biological membrane. The outer leaflet of the canalicular membrane is destabilized by bile salts, which are translocated in the canaliculus by BSEP (ABCB11). To dampen this effect, bile salts and phosphatidylcholine (PC) form mixed micelles with cholesterol translocated by ABCG5/G8. These mixed micelles have a lower capacity to extract lipids from the membrane. A second function of PC is the solubilization of cholesterol, which prevents the crystallization of cholesterol in the biliary duct and the formation of cholesterol gallstones. The bulk of PC is reabsorbed in the intestine and returns to the hepatocyte within the enterohepatic cycle. However, the half-time of PC to flip spontaneously from

the inner to the outer leaflet of a lipid bilayer is very low; therefore PC must be translocated across the membrane of the hepatocyte by an active transporter. The multidrug resistance protein 3 (MDR3), also called ABCB4, is localized only in the canalicular membrane of the hepatocyte (▶Fig. 2.1) and is indispensable for the primary active transport of PC from the inner to the outer leaflet of the canalicular membrane against a concentration gradient. The mouse homologue is called Mdr2 and fulfills the same function as MDR3 to flop PC across the apical membrane of hepatocytes. Mutations in the MDR3 gene caused different types of liver diseases, such as progressive familial intrahepatic cholestasis type 3 (PFIC3), intrahepatic cholestasis of pregnancy (ICP), and low phospholipid–associated cholestasis (LPAC).

2.3.1 A brief history of MDR3

During an analysis of cDNAs from human liver in 1987, van der Bliek et al. identified a gene that is highly homologous to the human P–gp and designated it *MDR3*. One year later the complete cDNA sequence was published (67). This sequence is composed of two similar halves. One half consists like BSEP of six putative TMHs and one NBD. The NBDs are identical to those of the human MDR1. Furthermore, the TMDs showed up to 80% identity. Divergence between MDR1 and MDR3 is greatest at the N-terminus and in the 60–amino acid linker connecting the two halves (67).

While MDR1 transports a wide variety of structural unrelated substances and is involved in multidrug resistance (MDR), no drug-pumping activity has been demonstrated for MDR3 (68). Smit and coworkers characterized mice with a disruption of *mdr2* in 1993. They ascertained that the homozygous disruption of the murine homologous *mdr2* gene leads to a complete absence of PC and cholesterol from bile (69). Furthermore, mice heterozygous for *Mdr2* (Mdr2$^{-/+}$) have normal amounts of cholesterol and only 40% of PC in bile. Human MDR3 can functionally replace *mdr2* in knockout mice (70). This demonstrates that the closely related Mdr2 and MDR3 carry out the same function. Direct evidence that MDR3 can translocate endogenous PC has been obtained in enhanced transport of newly synthesized [^{3}H]choline-labeled PC to the surface of transgenic fibroblast (71). This suggested that MDR3 translocates specifically PC from the inner to the outer leaflet of the canicular membrane.

Van Helvoort and coworkers (72) were the first to demonstrated specific transport of a short-chain PC in polarized pig kidney epithelial cells transfected with MDR3. In this study they measured lipid translocation across the plasma membrane by extracting fluorescently labeled short-chain lipids from the cell surface into the basolateral and apical media. MDR3 translocated fluorescently labeled PC but not the other lipid analogues (72). However van Helvoort et al. showed that radiolabeled short-chain PC lacking the fluorescence moiety was not translocated into the apical medium by MDR3.

2.3.2 MDR3 – an ATP-binding cassette (ABC) transporter

The 141-kDa lipid translocase is postranslationally modified by glycosylation at two predicted asparagine residues (N91 and N97) and is allocated to the group of P glycoproteins based on amino acid sequence homology. Like BSEP, MDR3 is a so called full-size transporter and is encoded on one structural gene $(NBD\text{-}TMD)_2$ (see ▶Fig. 2.2).

2.3.2.1 Transport machinery – the flippase model

Two models, the "vacuum cleaner model" and the "flippase model", are postulated for the transport of hydrophobic substrates by ABC transporters. The vacuum cleaner model proposes that the molecule in the cytosol interacts with the transporter, enters a hydrophobic cavity of the ABC-transporter, and is pumped into the extracellular space.

In contrast, Higgins and Gottesman proposed a flippase model for mammalian P-glycoproteins (73). P-gp binds an amphipathic molecule located in the inner leaflet of the plasma membrane and flips the molecule to the exoplasmic leaflet. Therefore a substrate-binding site must be accessible from the lipid phase. The substrate accumulates in the outer leaflet, forming a concentration gradient between the cytosolic and exoplasmic leaflet of the plasma membrane. From the leaflet the substrate can freely diffuse into the extracellular medium. On the basis of the flippase model, it is feasible to explain the observation that PC secretion depends on the expression of Mdr2, the mouse homolog of MDR3, and the bile salt concentration (74). Elferink and coworkers showed if either PC or bile salts were lacking, PC would not be detectable in bile, concluding that bile salts translocation is the main driving force for the secretion of phospholipids (75).

It is assumed that P-gp, which is over 76% identical to MDR3, can bind substrates within the inner leaflet of the membrane as well as from the cytosol. How ABC transporters recognize and translocate substrates is still unclear and the subject of intensive investigation.

2.3.2.2 MDR3 – a drug ABC transporter?

MDR3 shares 78% amino acid sequence identity with the well-characterized drug-pumping ABC transporter P-gp. Because of the high amino acid sequence homology between MDR3 and MDR1 (over 85%) it was assumed that MDR3 also translocates drugs. However, initial experiments with MDR3 cDNA or its mouse homolog Mdr2 transfected cells showed no drug resistance (67,76–78) and MDR3 was not detected in MDR cell lines (67,79). The first indication that MDR3 translocates drugs was obtained by Kino et al. (80). They observed that MDR3 transfected yeast cells showed low-level resistance against the antifungal agent aureobasidin A. Another study of MDR3 was performed by Smith et al. (81), who investigated vectorial substrate transport by polarized pig kidney monolayers transfected with MDR3 cDNA of several MDR1 substrates. They observed that the transport of digoxin, paclitaxel, vinblastine, and ivermectine into the apical medium was significantly increased in the MDR3-transfected cells compared with the control cells. Digoxin transport by MDR3 was efficiently inhibited by the MDR1-specific inhibitor verapamil, cyclosporine, and PSC833, which also inhibited the transport of short-chain PC. Verapamil had also previously been shown to inhibit the translocation of short-chain C_6-NBD-PC (72,82). No significant transport of some other MDR1 substrates, such as cyclosporine or dexamethasone, was determined.

These results suggest that MDR3 is not specific for PC and is able to translocate various typical MDR1 substrates as well. But why is drug transport observed only in polarized monolayers transfected with MDR3 cDNA? Currently there is no satisfactory explanation. Further studies on the translocation of long-chain PC and drugs by MDR3 are required.

2.3.3 Analysis of the substrate specificity of the PC translocator

The analysis of lipid transporters is very complex by reason of the difficulty of developing a reliable assay for the molecular mechanism of lipid transporters. Following are described two different ways of analyzing the function of P-glycoproteins and especially lipid translocases. On the one hand, MDR3 translocates PC across the membrane; three different approaches to this have been reported. On the other hand, MDR3 hydrolyzes ATP. The resultant ATPase activity correlates indirectly with the substrate transport.

2.3.3.1 Transport of lipids and lipid analogues by the ABC transporter

Currently no sensitive assay for measuring naturally occurring long-chain protein-mediated lipid translocation from one leaflet to the other leaflet of the membrane exists. Nevertheless Sleight and Pagano used the lower hydrophobicity of short-chain lipids (C_5-C_6 acyl chain) to determine lipid transport of lipid translocases, which allows their free exchange as monomers via the aqueous phase (83). At first short-chain lipids are easily integrated into the surface of the membrane of interest and can be detected by a spin-, fluorescent-, or radiolabel on the short-chain. The transport can be measured by chemically quenching of the spin-labeled or fluorescent analogue in the outer leaflet (84) or by "back-exchange." To date two different systems to determine short-chain PC transport by MDR3 or the mouse-homologous Mdr2 have been described.

Ruetz and Gros expressed Mdr2 in the membrane of secretory vesicles obtained from a yeast secretion mutant (82). These vesicles can be easily isolated and consist of a pure population of inside-out vesicles, meaning that the cytoplasmic NBDs of the ABC transporter are located on the outside of the vesicle. To determine Mdr2-driven transport of PC from the outer leaflet into the inner leaflet of the vesicular membrane they used fluorescent-labeled short-chain PC – C_6-NBD-PC: (N-6[7-nitro-2,1,3-benzoxadiazol-4-yl]-amino-hexanoyl-phosphatidylcholine) – which is chemically reduced to the non-fluorescent compound by a membrane-impermeable reducing agent such as sodium dithionite. Dithionite reduces only the C_6-NBD-PC located in the outer leaflet, whereas the translocated C_6-NBD-PC in the inner leaflet remains unaffected. Detergent disruption of the vesicles lead to a decrease of fluorescence emission because of the release of translocated C_6-NBD-PC. With this system Ruetz and Gros proved indeed a very small but specific transport of short-chain PC analogue by Mdr2. Second, they showed that transport was ATP-dependent and inhibited by verapamil, a specific inhibitor for MDR1.

In the "back-exchange" method, short-chain lipids are extracted from the outer leaflet by bovine serum albumin (BSA). BSA has the ability to selectively bind short-chain lipids from the outer leaflet. The lipids are analyzed by two-dimensional thin layer chromatography (TLC) and the transport activity is calculated by the ratio between translocated and total amount of short-chain PC (85).

The floppase activity of MDR3 was confirmed by van Helvoort et al. using LLC-PK1 pig cells transfected with an MDR3 cDNA construct (72). LLC-PK1 cells are able to grow as monolayers on filters and MDR3 is found only in the apical membrane. Cells are cultured in the presence of a short-chain lipid precursor, which is taken up and converted into the corresponding short-chain lipid analogue. The intracellularly synthesized C_6-NBD-PC was specifically transported by MDR3 but not C_6-NBD-phosphatidylethanolamine, C_6-NBD-sphingomyelin, or C_6-NBD-glucosylceramide. Remarkably, radiolabeled short-chain PC with two C_8 fatty acids lacking the fluorescence moiety

(C_8C_8-[^{3}H]PC) were slightly translocated into the apical medium. Van Helvoort and colleagues confirmed that the high specificity of MDR3 is determined by the choline head group.

To date, only Smith and coworkers have been able to generate a system for the translocation of long-chain PC through the membrane of fibroblasts from transgenic mice by MDR3 (71). Intracellular synthesized radioactively labeled PC is inserted into the inner leaflet and translocated to the outer leaflet in the presence of MDR3. PC-TP, a PC-specific transfer protein, carries out the exchange of labeled PC from the outer leaflet to acceptor liposomes in the medium. In this study Smith et al. determined an increased translocation of long-chain PC in the presence of MDR3. One main drawback of this system is the high background in the absence of MDR3 by vesicular transport. This makes usage of this assay extremely complicated.

Thus far no in vitro system for the translocation of PC by MDR3 is established because of the challenge of cloning, expressing, and purifying functional MDR3 in sufficient amounts and the technical difficulty of measuring the translocation of natural PC.

2.3.3.2 Substrate-stimulated ATPase activity

ABC transporters hydrolyze ATP to energize the transport across the membrane. Since ATP hydrolysis is linked by substrate translocation, the transport activity can be visualized indirectly. Most ABC transporters offer a basal ATPase activity. This ATPase activity is stimulated or inhibited by adding the substrate or inhibitor. There are two assays for measuring the ATPase activity by the determination of released inorganic phosphate: the malachite green assay (86) and the NADH-coupled assay (87,88). Both assays measure the release of free orthophosphate.

The highly sensitive malachite green assay is based on the complex formation of free phosphate with molybdate. The reaction of phosphomolybdate and the dye malachite green results in a green complex, whose absorbance can be easily determined at a wavelength of 620 to 650 nm. Nevertheless, a disadvantage of this method is its inability to observe the hydrolytic reaction continuously. The NADH-coupled assay enables one to follow the rate of ATP hydrolysis in real time by coupling the release of P_i and the oxidation of NADH to NAD^+. The ATPase hydrolyzes ATP to ADP and P_i. ADP is converted to ATP and phosphoenolpyruvate (PEP) to pyruvate by pyruvate kinase. The lactate dehydrogenase reduces pyruvate to lactate, while NADH is oxidized to NAD^+. The decrease of NADH is then determined at a wavelength of 340 nm.

The precondition to measuring ATPase activity is simple: sufficient expression of MDR3. To date it has not been possible to clone and express functional MDR3 in bacterial systems such as *E. coli* or *L. lactis* because of the "toxic" or "unstable" DNA sequence (as described in section 2.2.5). The expression of MDR3 in mammalian cell lines such as LLC PK1 and insect cells has been demonstrated by different groups (72,82). However, the obtained protein amounts are not sufficient to purify MDR3. Thus, up to now, it has not been possible to measure the PC-stimulated ATPase activity of membrane vesicles containing MDR3 and/or of isolated MDR3 in detergent solution or reconstituted into liposomes. To overcome this major obstacle it is crucial to study MDR3 in vitro and obtain a more detailed knowledge of this interesting ABC transporter as expressed inside the liver.

2.4 Structure and function of the breast cancer resistance protein (ABCG2; BCRP)

2.4.1 History of ABCG2

ABCG2 was first identified in human (BCRP, ABCG2) carcinoma cells. Despite the absence of overexpression of known multidrug transporters, like P-gp or MRP1, these cells displayed a remarkable resistance to multiple chemotherapeutic drugs such as doxorubicin and mitoxantrone. The gene conferring this resistance was isolated and subsequently used to transfect carcinoma cells, which then displayed a diminished accumulation of daunorubicin in flow cytometry assays. Additionally, this transport function appeared to depend on the presence of ATP, and this transport protein was termed breast cancer resistance protein (BCRP; ABCG2) (89). Independently, ABCG2 was discovered as the determinant responsible for the resistance of human colon carcinoma cells selected in mitoxantrone. Isolated cDNA clones displayed high levels of resistance to mitoxantrone. The gene showed relation to the *Drosphila melanogaster* white gene and homology to ABC transporters; it was named MXR for "mitoxantrone resistance" (90). Furthermore, ABCG2 was identified among a group of new human ABC transporters that were found to be highly expressed in the placenta. The isolated cDNA contained an open reading frame of 655 amino acids consisting an ABC half-size transporter with an N-terminal NBD and a C-terminal TMD (91). Although it was discovered three times in different contexts, the gene involved always encoded ABCG2.

2.4.2 Structure and function of ABCG2

ABCG2 is a 72-kDa 655–amino acid glycoprotein. Among the members of the ABC transporter family, ABCG2 has, like other members of the ABCG subfamily, a reverse topology, meaning that the NBD is located N-terminal to the TMD. With only one NBD and one TMD encoded on a single gene, ABCG2 is considered to be a half-size transporter and thought to dimerize to become a functional ABC transporter (see ▶Fig. 2.2). Several studies have focused on this oligomerization behavior. Interestingly, intermolecular disulfide bonds are required to obtain a dimeric protein. Cysteine scanning mutagenesis revealed that residue C603 of ABCG2 is involved in intermolecular cross-linking via disulfide bonds (92). Additionally, no mutation of any other cysteine residue had an effect on the dimerization of ABCG2 or its activity. In agreement with these results, Henriksen et al. showed that the oligomeric species of ABCG2 was observed with the use of a nonreducing SDS-PAGE can be gradually disrupted by the addition of a reducing agent (93). Here, mutational analysis of the three cysteine residues located in the third extracellular loop showed that only the C603A mutant impaired dimerization. However, a cell survival assay with mitoxantrone showed that this mutation was still as resistant as the wild type, indicating that the disulfide bond is not essential for the transport function. A biotinylation assay supported the idea that the other two cysteine residues in this loop, C592 and C608, form an intramolecular disulfide bond. However, this disulfide bond is important for protein degradation (94).

An important feature of ABCG2 is the GXXXG motif, which has been identified as a recurring transmembrane sequence and is proposed to be an interaction site between the transmembrane α-helices of different monomers. Polgar et al. investigated the only putative GXXXG motif in transmembrane helix 1 of ABCG2. Mutation of one or both of

the glycine residues resulted in lower ATP hydrolysis and a reduced substrate transport rate, although the protein was still expressed at similar levels on the cell surface. These findings support the hypothesis that the GXXXG motif plays a role for the correct orientation of the transmembrane segments toward each other in the functional transporter. Mutational studies of G553 indicate an involvement of this residue in the dimerization of ABCG2 (95).

Another important characteristic of ABCG2 is its hyperglycosylation, deduced from the apparent molecular weight of the protein in SDS PAGE gels and susceptibility to PNGaseF treatment. The glycosylation, however, appears to have no influence on the trafficking of ABCG2 to the plasma membrane. Surface expression was investigated by immunostaining of human ovarian carcinoma cells and hamster ovary cells. Although three glycosylation sites are predicted to be potentially located in the third extracellular loop, only the N569Q mutant showed impaired glycosylation. As mentioned in section 2.2.5, this impaired glycosylation does not result in misstrafficking, in contrast to, for example, the N557 alanine mutation, which results in a ER localization of ABCG2 (96).

Nonglycosylated ABCG2 still showed reduced accumulation of the substrate rhodamine 123 in flow-cytometric assays and normal ATPase activity, which can be stimulated by prazosin. The results were comparable to levels found for glycosylated ABCG2 in crude membrane preparations, indicating that glycosylation is not essential for the function of ABCG2. Many studies investigating the function of ABCG2 have employed mutagenesis to clarify the role of different residues in the protein. Residues C592, C603, and C608 are involved in intra- or intermolecular disulfide bonds and N596 is glycosylated. Furthermore, residue R482 has been extensively characterized. Early isolates of ABCG2 from carcinoma cell lines showed a mutation at this position. By testing the accumulation of rhodamine 123 in cells expressing the variants R482G and R482T, broader substrate specificity was observed (97). Whereas the wild-type protein conferred no resistance to compounds like rhodamine 123, doxorubicin, or daunorubicin, expression of ABCG2 and the mutants R482G and R482T reduced the accumulation of the drugs and prolonged cell survival in cytotoxicity assays. Other compounds – like mitoxantrone, prazosin, and Hoechst 33342 – are substrates for both mutant and wild-type transporters (98,99) . A later study confirmed previous results and additionally observed binding of substrates, which are not transported to the wild-type transporter (100).

A common single-nucleotide polymorphism encoding the mutation Q141K is linked with the occurrence of gout. ABCG2 was shown to be located in the brush-border membrane of kidney proximal tubule cells. Functional assays with *X. laevis* oocytes expressing wild-type ABCG2 or Q141K mutant showed that the latter exhibited urate efflux, thereby linking ABCG2 to this genetic disease (101). A recent study revealed that this mutant is exhibiting increased susceptibility for lysosomal and proteasomal degradation (102).

2.4.3 Analysis of the substrate specificity of ABCG2

Owing to the discovery of ABCG2 in drug-resistant cells, the first reported substrates for it were predominantly chemotherapeutic drugs. These included mitoxantrone, flavopiridol, metothrexate, irinotecan and its active metabolite SN-38, porphyrines, and tyrosin-kinase inhibitors such as imatinib and gefitinib (103). Other substrates are antibiotics (104,105), flavonoids, antivirals (106,107), folic acid (108), and fluorescent dyes such

as Hoechst 33342. Mutation of the arginine residue at position 482 conveys a broader substrate spectrum including rhodamine 123 and anthracyclines such as doxorubicin. Because of its broad substrate spectrum and its expression in several tissues apart from the liver – such as the small intestine, colon, central nervous system, testis, ovary, and placental syncytiotrophoblasts – the transporter is thought to have a protective role (103,109,110). The number of ABCG2 inhibitors identified is equally large. Fumitremorgin C was the first inhibitor described (111). Its analog, Ko143, was found to be one of the most effective ABCG2 inhibitors (112). Some inhibitors were also inhibitors of P-gp or MRP – among them cyclosporine (113) and elacridar (GF120918)(114). Many compounds are both inhibitors and transported substrates, such as dihydropyridines (115). Despite the great number of substrates and inhibitors described to date, no clear structural requirements for a binding compound could be identified.

2.4.4 Expression, purification, and biochemical studies of ABCG2

To date, ABCG2 has been successfully expressed in a number of different vector systems and host organisms. Early studies have been done with drug-selected mammalian cell lines. Finally, the isolation of the cDNA offered the opportunity to move the expression to some heterologous hosts, such as *Xenopus* oocytes, insect cells, yeast, or bacteria.

Baculovirus-infected insect ovary cells (*Sf*9) and High Five cells offer an alternative to mammalian cell lines and have been successfully used to overexpress ABCG2, although in both cases hypoglycosylation, transport, and ATPase activity were observed (116,117). Other expression systems include yeasts like *P. pastoris* and *S. cerevisiae*. Mao et al. expressed ABCG2 in *P. pastoris,* obtaining active protein comprising about 3% of the total protein in microsome preparations (118). Similar expression levels could be observed in baker's yeast, yielding protein with ATPase activity, which could be stimulated by substrate (119). Additionally, a prokaryotic expression system has been reported employing the gram-positive bacterium *L. lactis* (120). Expression in another bacterial system, *E. coli,* did not yield functional protein (121).

Especially for the purification of ABCG2 from the membrane fraction of the expression host, a high yield is needed. Protein expression of the systems mentioned previously in this section was tested on the ability to obtain high yields of ABCG2 after purification. Solubilization of ABCG2 using different detergents showed the best results with the use of lysophosphatidylcholine (LPC) and n-dodecyl-β-D-maltoside (β-DDM) for *P. pastoris* membranes and FosCholine-14 and -16 for ABCG2 expressed in insect cells (122,123). Also used for solubilization of protein from insect cell membranes was CHAPS (117). Purification steps of the amino-terminal-histidine-tagged protein in all cases yielded sufficiently pure protein after immobilized metal-ion affinity chromatography (IMAC). Because of weak binding to the affinity resin, further purification steps were necessary when the insect cell expression system was used. These included ion exchange and size exclusion chromatography (117). ABCG2 retained ATPase activity and substrate binding after its purification.

Because of its ability to efflux a broad variety of substrates, multiple drug binding sites have been proposed for ABCG2. Clark et al. investigated this with heterologous displacement assays. [^{3}H]daunomycin binding constants were measured in the presence of other known substrates of the ABCG2 gain-of-function mutant R482G. Three distinct binding sites were proposed, which are interlinked by allosteric communication (124). Several

studies of the substrate specificity and drug binding could be obtained by employing fluorescent substrates of ABCG2. For example 1,4-dihydropyridines could be identified as ABCG2 substrates by photoaffinity labeling with [^{125}I]Iodoarylazidoprazosin (IAAP) and [^{3}H]azidopine (125).

Since the translocation process is ATP-dependent, the ATPase activity of ABCG2 has been measured to confirm its physiologic activity. The majority of kinetic parameters were obtained on membrane preparations containing other ATPases. The wild type and the R482G isoform are capable of hydrolyzing ATP in the absence of any substrate (98). Since the influence of substrates and inhibitors on hydrolytic activity is an indicator of interaction with the protein, the measurement of ATPase activity is the focus of several studies. ATPase activity has been used as a readout in order to identify cholesterol content of the membrane as a major factor in ABCG2 activity (126). Cholesterol loading and depletion experiments showed stimulation of ATPase activity by substrates and improved drug transport in cholesterol-loaded membranes. In contrast, ATPase activity could not be stimulated in cholesterol-depleted membranes, indicating an essential role of membrane cholesterol.

Another tool to gain further knowledge about the topology of ABCG2 is epitope insertion mutagenesis. One study employed hemagglutinin (HA) tags to probe the predicted hydrophilic regions of ABCG2 via immunofluorescence (127). The results supported a model of six transmembrane helices with the amino and carboxy termini located intracellularly. A later study investigated a current homology model of ABCG2 by epitope insertion and found significant differences in the location of the predicted transmembrane segments (122).

To date there are no high-resolution structural data on ABCG2, although some attempts to obtain such data have been undertaken. These include negative-stain electron cryomicroscopy of purified protein. ABCG2 overexpressed in insect cells was solubilized and retained its stimulated ATPase activity. Analysis of the electron microscopy data revealed large particles (~170 Å in diameter) with a noticeable fourfold symmetry, in agreement with a higher oligomer as postulated by biochemical analysis. The final three-dimensional structure with an estimated resolution of ~18 Å could be accurately fitted with homology models of ABCG2, forming a tetramer. Data from size-exclusion chromatography and blue native PAGE supported the idea that ABCG2 forms a higher-order oligomeric species under the tested conditions (122).

Rosenberg et al. used purified ABCG2 expressed in *P. pastoris* to obtain two-dimensional crystals. The substrate mitoxantrone had a noticeable effect on the crystal shape. Analysis showed a significant change in unit cell dimensions, indicating a conformational change upon drug binding. A new homology model verified by epitope insertion mutagenesis supported the structural data by showing rigid body motion of two transmembrane helices, leading to a more compact conformation of the transporter in the drug-bound state. However, a three-dimensional structure is still a long way off. Eventually that will provide a detailed look at the function of ABCG2 at the molecular level.

2.5 Concluding remarks

Several interesting transporters are expressed within the hepatocytes, contributing to the function of these cells. This chapter focused on three of them, BSEP, MDR3, and

ABCG2. A wealth of information is to be derived from studies of their expression and mutation in different mammalian cell lines – studies focusing on their localization, trafficking, and activity. Although such studies have revealed extremely valuable and often essential information, the next step must be to achieve a molecular understanding of these transport mechanisms. Here, the first prerequisite is to elucidate the overexpression of these transporters, which will lead to their characterization directly in isolated membranes and/or after subsequent solubilization and purification in detergent solution. As described and summarized, the expression of membrane proteins is by no means trivial and often hampered by a too low expression in homo- or heterologous expression systems. However, if overexpression can be achieved for BSEP, MDR3, or ABCG2, the gain in knowledge derived from localization studies as well as mutational analysis will shed much light on the molecular mechanism of transport of a large variety of substrates with ATP only as an energizing molecule. Truly it will be a long way to go, but the information obtained will be worth the effort.

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2.2. Chapter 2 – Recombinant Production of ABCG2 in *Escherichia coli*

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- Cloning of variants and mutants
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List of Abbreviations

ABCG2, ATP-binding cassette transporter subfamily G member 2; CHS, cholesteryl hemisuccinate; FC-16, Fos-Choline-16; IMAC, immobilized metal ion affinity chromatography;

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ABSTRACT

ABCG2 is a transporter in many barrier-forming tissues such as placenta, liver or blood-brain barrier. Due to its wide substrate spectrum ABCG2 is involved in the multidrug resistance of cancer cells and pathogenesis of gout and other diseases. Here, we describe the high-yield heterologous expression of the membrane protein ABCG2 in *E. coli.* A detergent screen determined Fos-Choline-16 as the most efficient choice for solubilization. Addition of the cholesterol analogue cholesteryl hemisuccinate enabled the purification of ATPase active ABCG2 from *E. coli* membranes. Here, we present the first overexpression system for ABCG2 in *E. coli* yielding ATPase active transporter.

INTRODUCTION

Cancer resistance to chemotherapeutic drugs is one of the main obstacles in successful cancer treatment (*1*). The ATP binding-cassette (ABC) transporter ABCG2 was, after P-gp (ABCB1) and MRP1 (ABCC1), the third identified contributor associated with multidrug resistance by efflux (*2, 3*). ABCG2 has a broad substrate spectrum of structurally divers organic compounds. The transporter is expressed highly in tissues involved in absorption, secretion or distribution, such as the intestine, liver and blood-brain barrier and is therefore attributed with a protective function (*4*). Due to its role in multidrug resistance its effects on pharmacokinetics have been studied extensively. ABCG2 is also capable of transporting various endogenous compounds including urate, porphyrins, riboflavin, estrogens and bile salts giving it a role in their cellular homeostasis and in the occurrence of gout (*5-7*).

ABCG2 belongs to the subfamily of ABC half-transporters and is comprised of one nucleotide binding domain (NBD) followed by a transmembrane domain (TMD). Consequently, the transporter is thought to rely on dimerization since a functional ABC-transporter consists of two NBDs and TMDs each (*7-9*). Posttranslational modification of ABCG2 includes N-linked glycosylation in the third extracellular loop and two disulfide bonds in the same loop, one intra- and one intermolecular. While none of these modifications are essential for the transporter's activity they influence its stability and half-life (*10, 11*).

Molecular investigation requires large amounts of pure protein, which can usually only be achieved by heterologous overexpression systems. To this end several expression systems have been established for ABCG2. For structural and functional investigation insect cells and the yeast *Pichia pastoris* have been the predominant source of recombinant ABCG2 (*12, 13*). Another common system, the rapid and inexpensive overexpression in *E. coli,* could not produce functional protein in the one approach published by Pozza et al. (*14*).

Here, we present the production of recombinant ABCG2 in *E. coli* and a purification protocol involving cholesteryl hemisuccinate to obtain functional ABCG2.

MATERIALS AND METHODS

Materials

Chemicals were purchased in analytical quality at Merck (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany), VWR (Radnor, PA) or Sigma-Aldrich (Munich, Germany). Detergents were obtained from Anatrace (Maumee, OH).

Constructs

The codon-optimized plasmid pQE-T7-ABCG2 for expression in *E. coli* was purchased from Qiagen (Hilden, Germany). ABCG2 cDNA of human origin was purchased from Source Biosciences (Nottingham, UK).

Strains

E. coli strains used in this study are summarized in Table 1

Cloning of ABCG2

The pQE-T7-ABCG2 construct codes for the transporter with an N-terminal hexahistidine fusion. The tag was extended by PCR to a decahistidine tag. The *E. coli*-optimized coding sequence of ABCG2 was subcloned into the pBAD/His A vector (Invitrogen, Carlsbad, CA) by Infusion reaction (Clontech, Mountain View, CA), which puts the expression under the control of the arabinose-inducible araBAD promotor,. To obtain a catalytically inactive ABCG2 two point mutations were inserted by PCR (E211Q, H243A). The primers used for these procedures are listed in the order of use in Table 2.

Expression of ABCG2 in *E. coli*

LB medium (10 g/l tryptone/peptone from casein, 5 g/l yeast extract, 5 g/l NaCl) supplemented with antibiotics was inoculated with a plasmid carrying strain. The main culture was started at an OD_{600} of 0.09 by adding an overnight starter culture. The culture was incubated at 37°C with shaking until an OD_{600} of 0.6 was reached. Cultures were cooled to 18°C and expression was induced by the addition of 1 mM IPTG. After incubation for 4 hours at 18°C cells were harvested (4000g, 20 min, 4°C) and stored at -20°C. Samples taken during induction were analyzed by SDS-PAGE

and immunoblot. Expression conditions were optimized by varying parameters including expression strain, temperature during induction and length of induction.

Purification of heterologously expressed ABCG2

Heterologously expressed protein was purified by immobilized metal ion affinity chromatography (IMAC). *E. coli* cells were suspended in 2 ml of lysis buffer (50 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA pH 8, protease inhibitor) per gram wet cell weight. The cell suspension was then lysed by two passes through a MP-100 Microfluidizer (Microfluidics, Westwood, MA) at 1.3 kbar. Cell debris was removed by differential centrifugation at 2000g, 15 min, 4°C and 14.000g, 30 min, 4°C. Membranes were sedimented by ultracentrifugation at 100000g, 1 h, 4°C. The membranes were suspended in membrane buffer (50 mM Tris pH 7.5, 100 mM NaCl, 20 % glycerol) to a protein concentration of 10 mg/ml. ABCG2 was solubilized with 1 % detergent for 1 h at room temperature. For optimization 0.2 % cholesteryl hemisuccinate was added during solubilization. Aggregates were removed by centrifugation at 100000g, 45 min, 4°C. The supernatant was applied to a HiTrap Chelating HP column (GE Healthcare, Freiburg, Germany) for affinity purification, washed (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM imidazole, 10 % glycerol, 0.0016 % FC-16) and eluted in IMAC buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 10 % glycerol, 250 mM imidazole, 0.0016 % FC-16). Protein was concentrated and the buffer exchanged (50 mM Tris pH 7.5, 100 mM NaCl, 10 % glycerol, 0.0016 % FC-16) with Amicon centrifugal filter units with a molecular weight cut-off of 50,000 Da (Merck KGaA, Darmstadt, Germany). For analysis, proteins were separated in discontinuous polyacrylamide gels under denaturing conditions. Immunoblots were performed with the anti-ABCG2 (6D171, Santa Cruz Biotechnology, Dallas, TX) and anti-Mouse-HRP antibody (Dianova, Hamburg, Germany).

Determination of ATPase activity by malachite green assay

The malachite green assay for determination of free phosphate was used as described (*15*). ATPase activity was measured in 100 µl volume containing 10 µg of solubilized protein, 5 mM $MgCl_2$ and ATPase buffer (50 mM Tris pH 7.5, 100 mM NaCl, 10 % glycerol). The reaction was started with addition of 1 mM ATP. 25 µl of the reaction was stopped in 175 µl 10 mM sulfuric acid at defined time points. 50 µl of dye

solution (0.096 % (w/v) malachite green, 1.48 % (w/v) ammonium molybdate, 0.173 % (w/v) Tween-20 in 2.36 M H_2SO_4) was added and absorption at 420 nm determined after 30 min incubation.

RESULTS

To facilitate fast, inexpensive overexpression of ABCG2 we aimed for recombinant expression of the transporter in *E. coli*. To this end a codon-optimized ABCG2 cDNA was tested for expression in the prokaryote.

ABCG2 was produced in most of the tested conditions and migrated as a 65 kDa protein in reducing SDS-PAGE, which is characteristic for the non-glycosylated form of ABCG2 (Figure 1 A) (*16, 17*). Although we did not observe a complete growth arrest, which Pozza et al. encountered in their system (*14*), we saw that ABCG2 production did inhibit *E. coli* growth more strongly than other recombinant proteins.

One problem encountered early on was the degradation of ABCG2 in *E. coli*. The prokaryote produced high amounts of the full-length protein, but a significant amount of degradation products was observed during immunoblot analysis (Figure 1 B). During extended periods of induction at higher temperatures (> 4 hours) recombinant protein amounts decreased (Figure 1 B). The first approach to address this problem was to optimize expression conditions by lowering the temperature during induction, which increased the amount of ABCG2 per OD_{600} (Figure 1 B).

One possible reason for degradation was an overloading of the *E. coli* secretion machinery and subsequent aggregation of the membrane protein. The so-called Walker strains haven been selected for improved soluble expression of recombinant proteins (*18*). Sequencing by Wagner et al. later showed that they carried mutations in the *lac*UV5 promoter of the T7-RNA polymerase, which reduced transcription and therefore slowed down the production of recombinant protein (*18, 19*). Such a feature could also improve ABCG2 stability. The two tested strains C41 and C43, however, did not alter the amount of degradation products (data not shown). A second approach aimed to improve the insertion of the membrane protein into the inner membrane by use of the *E. coli* strain MC4100-444. Uller et al. observed a closer association of ribosomes with the secretion machinery in *E. coli* MC4100 after knock-out of trigger factor and SecB (*20*). The more efficient transfer of protein to the Sec complex might improve membrane insertion and reduce opportunity for aggregation of ABCG2. Optimized ABCG2 cDNA was cloned into the pBAD vector to be used in the MC4100 strain. We noticed that these measures were not able to reduce the

accumulation of degradation products in the cells, though (Figure 1 C). The initial ABCG2 construct was therefore used at reduced induction temperatures for further preparation of ABCG2.

McDevitt et al. presented an extensive solubilization screen of ABCG2 produced in insect cells, where the transporter proved resistant to most detergents (*21*). ABCG2 produced in the yeast *Saccharomyces cerevisiae* also preferentially solubilized with harsher, lipid-like detergents (*22*). We had similar difficulties to solubilize ABCG2. As reported previously for the eukaryotic membranes, only lipid-like detergents of the Fos-Choline family, particularly Fos-Choline-16, showed more than 50 % efficiency in solubilization of the transporter (Figure 2).

Solubilized ABCG2 was purified by immobilized metal ion affinity chromatography (IMAC). With the system presented here, ABCG2 was purified to more than 85 % homogeneity in one step (Figure 3). The yield was low due to weak binding to the affinity resin, which other investigators also encountered during affinity purification of the transporter (*21*). The remaining contaminants are mostly comprised of degradation products as already observed in the cell. Ultimately, we obtained >1 mg of purified protein per liter culture.

Initially, ABCG2 from *E. coli* did not display ATPase activity in detergent solution compared to the catalytically inactive mutant. Since the ATPase activity of wild-type ABCG2 is strongly dependent on the presence of cholesterol in the surrounding membranes a substitute molecule, the more water-soluble cholesteryl hemisuccinate (CHS), was added during solubilization (*23*). Solubilization efficiency was identical to the efficiency without CHS (data not shown). With the addition of CHS wild-type ABCG2 showed an ATPase activity that was about 40 nmol P_i/mg·min after subtraction of background activity from the catalytically inactive mutant (Figure 4). This value is in agreement with the activity of detergent-solubilized ABCG2 from other overexpression systems. Özvegy et al. for example obtained an average value of 43 nmol P_i/mg·min for wild-type ABCG2 from Sf9 insect cells compared to 2 nmol P_i/mg·min for the K86M mutant and Rosenberg et al. observed a basal activity of 20-60 nmol P_i/mg·min in detergent-solubilized ABCG2 from *Pichia pastoris* (*24, 25*).

DISCUSSION

ABCG2 is a multidrug transporter with a well described role in cancer resistance to chemotherapeutics and in the homeostasis of various endogenous substrates (*4*). Many aspects of investigation, for example approaches to the as yet unsolved high-resolution structure, require high amounts of pure protein.

In our study we approached one of the bottlenecks of *in vitro* investigation, the protein availability. We could express ABCG2 in high yields in *E. coli* and purify the protein to >85 % homogeneity in one step.

Lack of posttranslational modifications by the prokaryotic host should not be a problem in regard to ABCG2, since its glycosylation and disulfide bonds do not have an influence on the transporter's activity as shown by Diop et al. and Kage et al. (*17, 26*). However, we did observe an increased degradation of the transporter in its recombinant host, which may be due to the lack of posttranslational modification. Without glycosylation the transporter is less stable and more susceptible to degradation, but non-glycosylated ABCG2 retains its transport activity (*10*).

Another cause for increased degradation of ABCG2 during expression could be inefficient insertion of the transporter into the membrane. Although the initial problem of degradation could not be resolved by the presented approaches, ABCG2 could ultimately be obtained in amounts enabling *in vitro* investigations (1 mg purified protein per liter culture). To address the issue other approaches could improve folding and insertion of ABCG2 into the membrane. A low copy plasmid with a weaker promoter may help alleviate stress on the secretion machinery and promote correct membrane insertion. Addition of molecular chaperones or expression of chaperones could further reduce protein aggregation. Due to fast growth of *E. coli* and uncomplicated up-scaling the required protein amounts would still be easily obtainable.

As other investigators observed in eukaryotic systems (*21, 22*), ABCG2 preferentially solubilized with zwitterionic, lipid-like detergent, especially Fos-Choline-16. For ABCG2 produced in baker's yeast it was observed that, contrary to the OG- and DDM-solubilized transporter, the ATPase activity of FC-16-solubilized protein could not be stimulated by prazosin (*22*). It may be necessary to exchange FC-16 for a

milder detergent or reconstitute the transporter into liposomes to obtain optimal activity.

Preliminary dynamic light scattering analysis of detergent-soluble, purified ABGC2 indicated a monodisperse, higher-order oligomer of about 430 kDa, which agrees with previous analysis by different groups (*21, 25, 27*).

ABCG2 from *E. coli* was initially not active. Activity could be restored by adding the cholesterol analogue cholesteryl hemisuccinate during solubilization. The purified protein displayed an ATPase activity comparable to previously obtained results for detergent-soluble ABCG2 (*24, 25*).

In the present study we obtained recombinant, ATPase-active ABCG2 from *E. coli*. The transporter is now available for molecular investigation from this fast and inexpensive overexpression system.

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TABLES

Table 1 Strains of microorganisms used in this study

Name	Genotype	Reference
	E. coli	
BL21 (DE3)	*fhu*A2 [*lon*] *omp*T *gal* (λ DE3) [*dcm*] Δ*hsd*S λ DE3 = λ *sBamHIo* Δ*EcoRI-B int*::(*lac*I::P*lac*UV5::T7 *gene*1) *i21* Δ*nin*5	NEB, Ipswich, MA
BL21 (DE3)pLysS	*E. coli* B F^- *dcm omp*T *hsd*S(r_B^- m_B^-) *gal* λ(DE3) [pLysS Cam^r]	Agilent, Santa Clara, CA
MC4100-444	F^- [*ara*D139]$_{B/r}$ Δ(*arg*F-lac)169* &lambda$^-$ e14- *flh*D5301 Δ(*fru*K-*yei*R)725 (*fru*A25)‡ *rel*A1 *rps*L150(strR) *rbs*R22 Δ(*fim*B-*fim*E)632(::IS1) *deo*C1 Δ*tig* Δ*sec*B	(*20*)
C41	F^- *omp*T *gal dcm hsd*S_B(r_B^- m_B^-)(DE3)	(*18, 19*)
C43	F^- *omp*T *gal dcm hsd*S_B(r_B^- m_B^-)(DE3)	(*18, 19*)
DH5α	F^- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-	Invitrogen, Carlsbad, CA

Table 2 Oligonucleotides used in this study

Name	Sequence 5'-3'
10xHis_for	CACCATCACCATCACCATCACCATCACCATATGAAACAGATGAG
10xHis_rev	ATGGTGATGGTGATGGTGATGGTGATGGTGTTTCATATGTATATCTC
InfpBAD_for	CGACGATGACGATAAGG
InfpBAD_rev	GGTTAATTCCTCCTGTTAGC
InfB-G2_for	CAGGAGGAATTAACCTGTTTAACTTTAAGAAGGAGATATAC
InfB-G2_rev	TTATCGTCATCGTCGGTGGTGCTCGAGTCTTAC
mut E211Q_for	GCATTCTGTTTCTGGATGAACCGACCACCGGCCTGGATAG
mut E211Q_rev	CCAGGCCGGTGGTCGGTTCATCCAGAAACAGAATGC
mut H243A_for	CCGCACCATTATTTTTAGCATTGCCCAGCCGCGTTATAGCATC
mut H243A_rev	AAAGATGCTATAACGCGGCTGGGCAATGCTAAAAATAATGGTG

All oligonucleotides were purchased at MWG Biotech (Ebersberg, Germany).

FIGURES

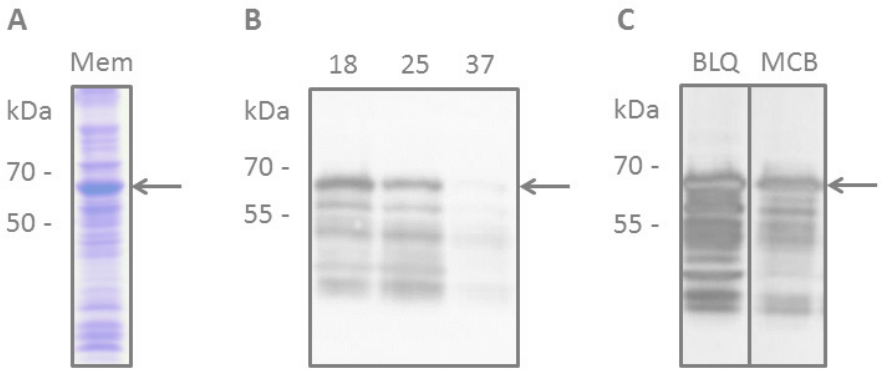

Figure 1 Expression of ABCG2 in *E. coli* BL21 (DE3) and MC4100-44

A ABCG2 expression in BL21 membrane preparations was analyzed by Coomassie-stained SDS-PAGE. **B** ABCG2 expression in whole cell lysates was monitored at different induction temperatures (18, 25 and 37°C). Samples after four hours of induction were analyzed by immunoblots developed with the 6D171 ABCG2 antibody. **C** Amount of ABCG2 degradation was compared during expression in BL21 (DE3) from the pQE-T7 plasmid (BLQ) and in MC4100-444 from the pBAD plasmid (MCB). Samples were analyzed with immunoblots developed with the 6D171 ABCG2 antibody.

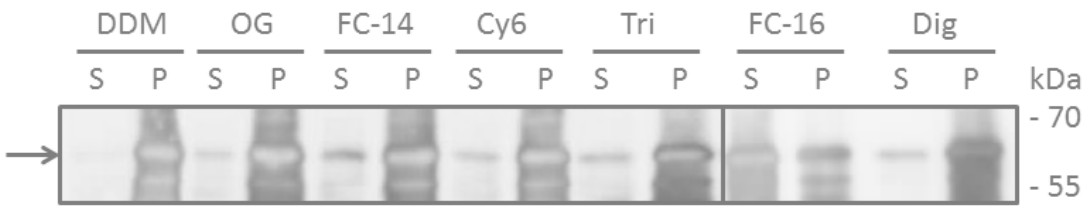

Figure 2 ABCG2 solubilization efficiency of different detergents

Solubilization of ABCG2 from *E. coli* membranes was tested with various detergents. A selection is depicted where soluble protein (S) was compared to the non-solubilized fraction after an one hour incubation (P). Tested detergents were dodecyl-β-D maltopyranoside (DDM), octyl-β-D-glucopyranoside (OG), Fos-Choline 14 (FC-14), 6-Cyclohexyl-1-Hexyl-β-D-Maltoside/Cymal 6 (Cy6), Triton X-100 (Tri), Fos-Choline-16 (FC-16) and digitonin (Dig).

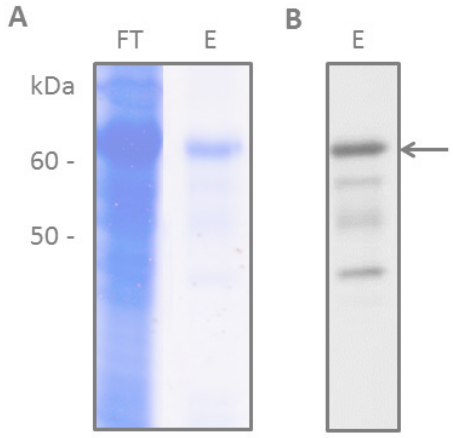

Figure 3 Purification of ABCG2 from E. coli by IMAC

ABCG2 produced in *E. coli* was purified by IMAC on a Ni^{2+}-loaded column. Flow-through (FT) and elution fractions (E) were analyzed by **A** Coomassie-blue stained gel and **B** immunoblot developed with ABCG2 antibody.

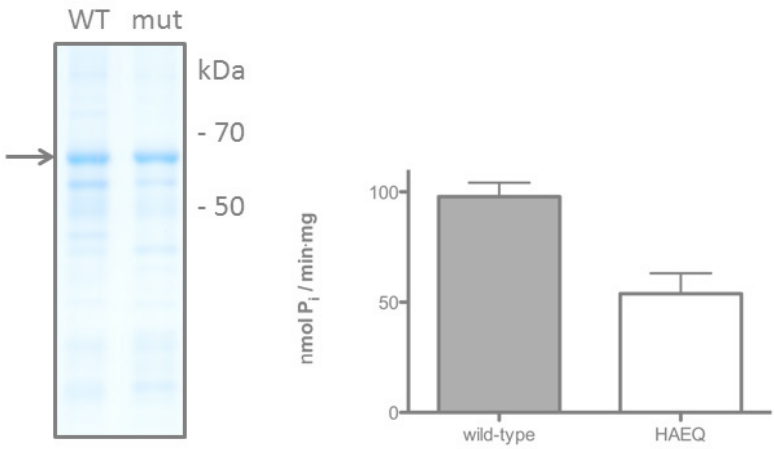

Figure 4 ATPase activity of ABCG2 purified from *E. coli*

ABCG2 wild-type (WT) and ABCG2 H243A E211Q (mut) were purified according to procedures detailed in the methods. The proteins were tested for ATPase activity by malachite green assay.

2.3. Chapter 3 - Posttranslational regulation of the bile salt export pump

Contribution to this publication: 70 %

- Expression in *P. pastoris*
- Optimization of protein purification
- Writing of the manuscript

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MEETING ABSTRACT Open Access

Posttranslational regulation of the bile salt export pump

Susanne Przybylla, Lutz Schmitt*

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Düsseldorf, Germany. 15-16 November 2013

Bile plays an essential role in the nutrient uptake of the human body. It consists mostly of amphipathic bile salts, phospholipids and cholesterol in mixed micelles. The bile solubilizes lipophilic nutrients, such as lipids and fat-soluble vitamins, and facilitates their uptake. The formation of bile depends on the activity of various ATP-binding cassette (ABC) transporters localized in the apical membrane of hepatocytes. The main components of bile, phosphatidylcholine, cholesterol and bile salts, are transported by multidrug resistance protein 3 (MDR3, ABCB4), ABCG5/G8 and the bile salt export pump (BSEP, ABCB11), respectively. BSEP, as the transporter of the main solute in bile, is considered to be the primary driving force of bile formation.

BSEP belongs to the family of ABC transporters, which constitute one of the largest protein families. They are primary active transporters with a common functional unit: two nucleotide–binding domains and two transmembrane domains. The nucleotide–binding domains fuel the transport by hydrolyzing ATP, while the transmembrane domains provide the translocation pathway over the membrane and are thought to be responsible for substrate recognition. Eukaryotic ABC transporters are usually encoded by one gene as in the case of BSEP (full-size transporters) or by two genes coding for one nucleotide-binding domain and one transmembrane domain each. In the latter case the transporter is called a half-size transporter and the two polypeptides homo- or heterodimerize to form a functional unit. An example for such a half-size transporter is ABCG5/G8. Mutations in ABC transporters can lead to severe diseases. For example an impairment of targeting or activity in BSEP leads to the accumulation of bile salts in the hepatocytes. Bile salts, in addition to their detergent function, can furthermore act as signaling molecules and thereby cause different forms of cholestasis, such as progressive familial intrahepatic cholestasis type 2 (PFIC2) or benign recurrent intrahepatic cholestasis type 2 (BRIC2).

An additional influence that can affect the development of diseases is the post-translational regulation of ABC transporter functionality. The regulation can occur directly in the form of chemical modifications or can be mediated for example by protein-protein interactions. For several ABC transporters of the apical hepatocyte membrane an influence of protein-protein interactions on targeting, turnover or activity could be shown.

MRP2 for example interacts with NHERF-1, a scaffold protein that is responsible for linking its interacting partners to the cytoskeleton. It has furthermore been reported that the interaction of MRP2 with NHERF-1 influences the apical membrane expression of MRP2. For example, NHERF-1 knock–out mice show reduced abundance of MRP2 in the membrane [1].

For BSEP two interaction partners are known to date. One of them is the AP2 adaptor related protein complex, which is involved in internalization of BSEP [2].

To study ABC transporters in their isolated form a heterologous overexpression system is required to obtain sufficient amounts of protein. Initially, toxicity of the BSEP cDNA for *Escherichia coli* was an obstacle. Therefore a *Saccharomyces cerevisiae* based cloning technique has been developed, which is independent of *E. coli* [3]. Subsequently, with the optimized cloning procedure an overexpression of BSEP could be established in the methylotrophic yeast *Pichia pastoris*. With the help of a GFP-fusion protein BSEP could be traced to the *P. pastoris* plasma membrane. Additionally, sucrose density centrifugation experiments confirmed the correct localization of BSEP. A subsequent detergent screen led to the identification of detergents suitable for BSEP solubilization. The transporter appears to be in a detergent-resistant environment as only the lipid–like,

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zwitter–ionic detergents of the Fos–choline and Cyclofos series, comparatively "harsh" detergents, could efficiently extract BSEP. Finally, a purification protocol was established to obtain BSEP in a detergent-solubilized form. With a dual–affinity tag tandem affinity purification could be employed to obtain the ABC transporter with approximately 75% purity [4].

In summary, the optimized cloning strategy for BSEP cDNA in *S. cerevisiae* as well as the efficient expression and purification protocols enable us to investigate the interaction of BSEP with other proteins *in vitro*.

Acknowledgments
We thank Dr. Philipp Ellinger and our collaboration partners Prof. Dr. Ralf Kubitz and Dr. Jan Stindt. Special thanks go to Dr. Diana Kleinschrodt and Iris Fey for stimulating discussions and support. This work is supported by the German Research Foundation (CRC 974).

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2.4. Chapter 4 – Analysis of the bile salt export pump (*ABCB11*) interactome employing orthogonal approaches

Contribution to this publication: 60 %

- Membrane Yeast-Two Hybrid Assay
- Data analysis
- Cloning of interaction partners and variants
- Expression in *P. pastoris* and *E. coli* and protein purification
- *In vitro* pull-down analysis
- Writing of the manuscript

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Analysis of the bile salt export pump (*ABCB11*) interactome employing orthogonal approaches

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Keywords

ATP-Binding Cassette Transporters; ATP-Binding Cassette Transporters: Regulation; Membrane Yeast Two-Hybrid; Co-Immunoprecipitation; Pull-Down Assay; Mass Spectrometry

FOOTNOTE PAGE

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List of Abbreviations

BSEP, bile salt export pump; PPI, protein-protein interaction; MRP2, multidrug-resistance-associated protein 2; MYTH, membrane yeast two-hybrid; MS/MS, tandem mass spectrometry; co-IP, co-immunoprecipitation; AP-2, adaptor protein complex 2; BACS, bile acyl-CoA synthetase.

Financial Support

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ABSTRACT

The bile salt export pump (BSEP, *ABCB11*) plays an essential role in the formation of bile. In hepatocytes, BSEP is localized within the apical (canalicular) membrane and a deficiency of canalicular BSEP function is associated with severe forms of cholestasis. Regulation of correct trafficking to the canalicular membrane and of activity is essential to ensure BSEP functionality and thus normal bile flow. However, little is known about the identity of interaction partners regulating function and localization of BSEP. In our study, interaction partners of BSEP were identified in a complementary approach: Firstly, BSEP interaction partners were co-immunoprecipitated from human liver samples and identified by mass spectrometry. Secondly, a membrane yeast two-hybrid assay was used to determine protein interaction partners using a human liver cDNA library. Interaction partners that were identified by membrane yeast two hybrid assays and MS were verified by *in vitro* interaction studies using purified proteins. **Conclusion:** By these complementary approaches, a set of eleven new BSEP interaction partners was identified. These include factors of the early secretory pathway, radixin and the bile acyl-CoA synthetase.

INTRODUCTION

One of the liver's major functions is the production of bile. During digestion bile facilitates the absorption of lipids and fat-soluble vitamins. Furthermore, it serves as the main route for excretion of cholesterol and lipophilic waste products.

The bile salt export pump (BSEP, *ABCB11*) is an ATP binding cassette (ABC)-transporter located in the apical membrane of hepatocytes, which is indispensable for bile flow, as it translocates conjugated bile acids from the cell lumen into the bile canaliculus, driving bile salt-dependent bile flow (1). At the canalicular membrane BSEP is accompanied by other ABC-transporters, which transport additional bile components, such as the multidrug resistance protein 3 (MDR3, *ABCB4*), which is a lipid floppase specific for the phosphatidylcholine family, and the cholesterol transporter ABCG5/8 (2). These three compounds, the main organic constituents of bile, form mixed micelles in the canaliculus, which mitigate the toxic detergent effect of bile salts on the surrounding hepato- and cholangiocellular membranes and thereby preserve their integrity (3).

Dysfunction of BSEP with respect to expression, trafficking or function is often a molecular determinant of cholestasis. Reduced expression of BSEP at the canalicular membrane or impaired activity leads to accumulation of bile salts in the hepatocyte, which is a cause for benign recurrent intrahepatic cholestasis type 2 (BRIC2) or progressive familial intrahepatic cholestasis type 2 (PFIC2) (4, 5). Due to their detergent character, increased levels of bile salts lead to cell damage, while their function as signal molecules impinges on cell metabolism at lower concentrations.

Due to its significance in cholestasis development, many aspects of expression as well as the functional regulation of BSEP have been extensively studied (2, 6). In this field, one less studied aspect is the posttranslational regulation of BSEP by protein-protein interaction (PPI), which is, among other functions, a prerequisite for efficient trafficking and localization of BSEP to the canalicular membrane. Some PPIs, which influence the abundance of the ABC-transporter in the apical membrane have already been identified. For example, the adaptor protein complex 2 (AP-2) and HAX-1 take part in the endocytic retrieval from the canalicular membrane and the myosin II

regulatory light chain (MLC2) has been shown to influence anterograde trafficking of the transporter to the apical membrane (7-9). PPI also plays a role in stabilization of membrane proteins by tethering them to the cytoskeleton. One example is the multidrug-resistance-associated protein 2 (MRP2, *ABCC2*), which depends on scaffolding and membrane-cytoskeletal cross-linking proteins such as the sodium-hydrogen exchanger regulatory factor-1 (NHERF1/EBP50), radixin and ezrin for efficient localization to the apical membrane (10-13). Additionally, PPI can directly influence the activity of an interaction partner. This emphasizes that more detailed information on the interaction network of BSEP is required to understand how its trafficking and activity are regulated.

In this study, full-length, human BSEP was used in a membrane yeast two-hybrid (MYTH) screen using a liver cDNA library to identify previously unknown interaction partners. In parallel, proteins associated with BSEP were identified by tandem mass spectrometry (MS/MS) after co-immunoprecipitation (co-IP) from human liver samples. As a third line of interaction studies, soluble interaction partners were verified by *in vitro* pull-down assays.

MATERIALS AND METHODS

Yeast Strain

The *Saccharomyces cerevisiae* strain NMY51 was used for the membrane yeast two-hybrid (Dualsystems Biotech, Schlieren, Switzerland; MATa his3Δ200 trp1-901 leu2-3,112 ade2 LYS2::(lexAop)$_4$ -HIS3 ura3::(lexAop)$_8$–lacZ ade2::(lexAop)$_8$ -ADE2 GAL4). Yeast cells were transformed using the lithium acetate method as described in Gietz et al. (14).

Construction of Plasmids for the MYTH Screen

For the bait construct full-length human BSEP cDNA (5) was cloned into the pBT3-C vector by homologous recombination. pBT3-C was linearized with the restriction endonucleases NcoI and SfiI. BSEP cDNA was amplified by PCR with the following primer pair
5'-CAAATACACACACTAATCTAGACGGCCATTAATGTCTGACTCAGTAATTCTTCGAAGTATAAAG-3' and
5'-CTTGATATCGAATTCCTGCAGATATACCCATGACTGATGGGGGATCCAGTGGT-3'. Homologous recombination was performed in the yeast strain NMY51 plated on synthetic defined drop-out medium (SD) lacking leucine. Positive clones were selected by colony PCR. After plasmid isolation and transformation of *E. coli* DH5α the construct was verified by sequencing.

MYTH Screen

The MYTH assay was carried out as described in the DUALhunter manual (Dualsystems Biotech). Briefly, the yeast strain NMY51 was transformed with the bait construct pBT3-C-BSEP and the functionality of the system with BSEP as bait was assessed employing the recommended controls. Following that, the bait was tested for self-activation with the empty prey vector pPR3-N. To screen for interaction partners NMY51 was transformed with the bait construct and subsequently with 36 µg of a human adult liver NubG-X cDNA library (Dualsystems Biotech; $1.5x10^6$ independent clones) for complete coverage. Clones grown on SD medium lacking leucine, tryptophan and histidine (SD-LWH) were re-plated on SD medium supplemented with 40 µg/ml X-Gal and lacking in addition adenine (SD-LWHAdX). Plasmids of blue colonies were isolated and amplified in *E. coli* DH5α. Yeast cells

harboring the pBT3-C-BSEP plasmid were retransformed with the prey plasmids to confirm the interaction. Interaction partners were checked for false positives by a bait dependency test with the SV40 large T antigen fused to an Ost4p membrane anchor as unrelated bait (DUALhunter manual, control plasmid pDHB1-largeT). Remaining candidates were sequenced and identified with the basic local alignment search tool (BLAST) (15).

Cloning of Putative Interaction Partners for Production in E. coli

cDNA of potential interaction partners identified in the MYTH were cloned into the pET-51b(+) vector (EMD Biosciences, Inc., Darmstadt, Germany) for expression in *E. coli*. cDNA was amplified by PCR with the addition of ZraI and KpnI restriction endonuclease sites and inserted into pET-51b(+) via these restriction sites. All constructs were verified by DNA sequencing.

Production of Putative Interaction Partners in E. coli

Full-length radixin and radixin$_{1-318}$ were produced in *E. coli* BL21 (DE3), the bile acyl-CoA synthetase (BACS) in *E. coli* Rosetta (DE3) pLysS. LB medium (10 g/l Tryptone/Peptone from Casein, 5 g/l yeast extract, 5 g/l NaCl) or in the case of radixin and radixin$_{1-318}$ LBN (10 g/l Trypton/Pepton from Casein, 2 g/l glucose, 29.2 g/l NaCl) was inoculated to an OD_{600} of 0.09 and grown to an OD_{600} of 0.6 at 37°C and 180 rpm. Protein production was induced by addition of 0.5 mM IPTG. The proteins were produced at 18°C for 20 h. After cell harvest (3000 *g*, 20 min, 4°C) the sediment was suspended in lysis buffer (50 mM sodium phosphate pH 7, 100 mM NaCl, 1 mM EDTA, 20 % (w/v) glycerol) supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland) and lysed by two passages through the Microfluidizer M-110P (Microfluidics, Westwood, MA) at 1.3 kbar. The lysate was cleared by centrifugation at 4000 *g*, 15 min, 4°C followed by centrifugation at 15000 *g*, 30 min, 4°C.

Purification of Interaction Partners

Protein interaction partners were purified by affinity chromatography. *E. coli* cell lysate was applied to Strep Tactin resin (iba GmbH, Göttingen, Germany) by gravity flow and washed with buffer (50 mM HEPES pH 7, 150 mM NaCl, 1 mM EDTA). Protein was eluted in elution buffer (50 mM HEPES pH 7, 150 mM NaCl, 1 mM

EDTA, 2.5 mM desthiobiotin) and concentrated with Amicon centrifugal filter units with a molecular weight cut-off of 10,000 Da (Merck KGaA, Darmstadt, Germany). Purified protein was flash frozen and stored at -80°C.

Expression of BSEP

BSEP was produced in the methylotrophic yeast *Pichia pastoris* (*Komagataella pastoris*) as previously published in Ellinger *et al.* (16). In brief, *Pichia pastoris* X-33 (Life Technologies, Carlsbad, CA) was transformed with the construct pSGP18-2µ-BSEP. The yeast was fermented according to the Invitrogen Pichia Expression Kit manual in a 15 liter table-top glass fermenter (Applikon Biotechnology, Schiedam, the Netherlands) in 5 l of basal salt medium with addition of 500 ml of 50% (v/v) glycerol. Feeding 500 ml of methanol during 28 h induced protein production. Approximately 800 g of wet cell mass was flash-frozen in liquid nitrogen and stored at -80°C until further use.

Purification of BSEP

Pichia pastoris cells were suspended in homogenization buffer (50 mM Tris pH 8, 50 mM NaCl, 0.33 M Sucrose, 1 mM EDTA pH 8, 1 mM EGTA pH 8, 0.1 M 6-aminohexanoic acid, 1 mM DTT) supplemented with protease inhibitor cocktail tablets (Roche). Cell disruption was performed with the Microfluidizer M-110P (Microfluidics) in three passes at 2 kbar. Cell debris was sedimented by differential centrifugation (1500 *g*, 15 min, 4°C; 14000 *g*, 20 min, 4°C). Crude membranes were obtained by ultracentrifugation at 120000 *g* for 1 hour at 4°C and suspended in membrane buffer (50 mM Tris pH 8, 50 mM NaCl, 20 % glycerol). After a second ultracentrifugation step, the membranes were resuspended in membrane buffer to a protein concentration of 10-20 mg/ml. Membranes equivalent to 30 g of wet cell weight were diluted to a protein concentration of 5 mg/ml as determined by Pierce Coomassie Plus Assay (Thermo Fisher Scientific Inc., Rockford, IL). Fos-choline 16 (Anatrace, Maumee, OH) was added to a concentration of 1 % (w/v) and proteins solubilized with rotation at 4°C for 45 min. Aggregates were sedimented by ultracentrifugation at 100000 *g*, 40 min, 4°C. The supernatant was applied to 1 ml of Calmodulin Affinity Resin (Agilent Technologies, Santa Clara, CA) and incubated with light agitation at 4°C for 30 min. The resin was washed with CBP binding buffer (50 mM Tris pH 8, 150 mM NaCl, 5 mM β-mercaptoethanol, 1 mM magnesium

acetate, 2 mM calcium chloride, 15 % (w/v) glycerol, 0.022 % (w/v) DDM). BSEP was eluted with CBP elution buffer (50 mM Tris pH 8, 150 mM NaCl, 5 mM β-mercaptoethanol, 2 mM EGTA, 15 % (w/v) glycerol, 0.022 % (w/v) DDM), stored at 4°C and used within the next 48 h. For removal of the affinity tags 200 µg of BSEP were incubated with 2 units of HRV 3C Protease (Thermo Scientific, Rockford, IL) for 4 h at 4°C. Protease and affinity tags were removed by incubation with 50 µl of Ni-NTA Agarose (Qiagen, Hilden, Germany) for 30 min at 4°C.

Pull-down Assay of BSEP with Interactions Partners

For pull-down assays, interaction partners were immobilized on a Strep-Tactin resin. After 30 min incubation at 4°C and washing with pull-down buffer (50 mM sodium phosphate pH 7, 150 mM NaCl, 20 % (w/v) glycerol, 0.022 % (w/v) DDM) detergent-solubilized, purified BSEP was added for another 30 min at 4°C. Complexes were eluted after 5 times washing and analyzed by immunoblotting. For detection of BSEP, the monoclonal F-6 antibody (Santa Cruz Biotechnology, Dallas, TX) was used, the Strep-tag II was detected by anti Strep-tag II monoclonal antibody (Merck KGaA, Darmstadt, Germany). Horseradish peroxidase-conjugated mouse monoclonal secondary antibody was purchased from Dianova (Hamburg, Germany).

Immunoprecipitation of BSEP From Human Liver

All steps were carried out on ice or at 4°C unless stated otherwise. Human liver samples were obtained from the noncancerous resection margin during liver metastasis resection and were immediately processed. Liver samples were cut into small pieces and lysed in homogenization buffer (50 mM Tris/HCl pH 7.4, 250 mM sucrose) with eight strokes in a tight-fitting dounce homogenizer. The crude homogenate was centrifuged for 10 min at 1000 *g*, and afterwards for 10 min at 3000 *g*. 20 µl of the wet pellet were solubilized in 1 ml IP solubilization buffer (1 % Triton X-100 or digitonin in 50 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 % glycerol, Roche protease inhibitor cocktail (EDTA-free)) overnight with slow overhead inversion. Insoluble material was removed by centrifugation at 13000 *g* for 10 min. For immunoprecipitation the monoclonal BSEP antibody (F-6) was used and naïve mouse IgG_{2a} served as control (Santa Cruz Biotechnology, Dallas, TX). 2 µg of antibody were added to 20 µl of protein A/G+ agarose slurry (Santa Cruz Biotechnology) and the mixture was incubated for 10 min

at room temperature with rotation. The preloaded agarose was washed three times with IP solubilization buffer. 1 ml of the solubilization supernatant was added to agarose-bound antibody, samples were incubated for 4 h with slow overhead inversion and centrifuged for 1 min at 1000 *g*. The resulting pellet was washed three times with IP solubilization buffer. Remaining buffer was removed by aspiration and pellets were stored at -20°C. For further analysis, the agarose pellet was incubated in 64 µl 3 % (w/v) SDS for 20 min, followed by addition of 16 µl 5x reducing sample buffer (100 mM Tris/HCl pH 6.8, 3.2 % (w/v) SDS, 40 % (v/v) glycerol, 0.02 % bromophenol blue, 10 % β-mercaptoethanol). After incubation at 65°C for 15 min samples were spun down briefly and 40 µl were subjected to SDS-PAGE and immunoblotting. Detection was performed with the following antibodies: anti-BSEP K24, which was raised as described previously (17), anti-Radixin (GeneTex, Irvine, CA), anti-Adaptin α (BD Biosciences, Franklin Lakes, NJ) or anti-SLC27A5 (Abcam plc, Cambridge, UK).

Mass Spectrometry

Following detergent removal, a tryptic digest of the co-IP pellet was subjected to shotgun tandem mass spectrometry. The analysis was carried out on an UHPLC-coupled Orbitrap Elite mass spectrometer by the Molecular Proteomics Laboratory at the Biologisch-Medizinisches Forschungszentrum (BMFZ) of Heinrich-Heine-University Düsseldorf as detailed earlier (18).

RESULTS

Three complementary methods identify a novel set of BSEP interaction partners

A full understanding of a protein's regulation in regard to function and cellular trafficking requires detailed knowledge of its interactome. Techniques such as yeast two-hybrid approaches, tandem affinity purification or immunoprecipitation, the latter two often combined with mass spectrometry, have been developed and optimized in recent years. However, each of these techniques has its own advantages and disadvantages (19). For example, in yeast two-hybrid systems the presence of protein tags can prevent interactions or produce false positive results, while mass spectrometry-based approaches lack some sensitivity towards transient interactions. To cope with these limitations and to advance the understanding of BSEP on a molecular level we used three complementary methods to identify interacting proteins of this bile salt transporter.

Firstly, the MYTH was used to screen for putative interaction partners that interact directly with BSEP. In contrast to conventional yeast two-hybrid systems (19, 20) the MYTH, which has been established by Stagljar et al., allows for screening of membrane proteins (21). A schematic diagram of the system is provided in Fig. 1. The MYTH assay is based on the re-association of two ubiquitin halves. The protein of interest (bait) is produced in yeast as a fusion protein with the C-terminal half of ubiquitin (Cub) and a transcription factor (LexA-VP16). The putative interaction partners (prey) are fused to the mutated N-terminal moiety of ubiquitin (NubG). Interaction of bait and prey cause the two ubiquitin halves to reconstitute into the so called split-ubiquitin, which is recognized by an endogenous ubiquitin specific protease. The transcription factor is released and activates three reporter genes, which enable yeast to grow on selective media (21).

For our study the assay was established with full-length, human BSEP as bait. BSEP cDNA was cloned into the pBT3-C bait vector, which adds the required ubiquitin moiety and transcription factor to the C-terminus of the protein for optimal accessibility of the fusion proteins.

Functionality of the MYTH system with BSEP was ascertained by control assays. The first assay activated the system independently of bait interaction and thereby confirmed BSEP expression (Supplemental Fig. S 1). The second control assay tested the BSEP construct for self-activation, which is a common complication in applying the MYTH. A low bait expression level, which was indicated for this system in the first control assay, is of advantage. BSEP did not show self-activation in our setup (Supplemental Fig. S 1).

To screen for interaction partners the BSEP expressing yeast strain was transformed with a human adult liver cDNA library. This library had a complexity of $1.5x10^6$ independent clones and allowed for the screening of liver proteins or protein fragments of up to 450 amino acids.

As in any genetic screening method, the obtained clones contained a number of false positives. To reduce their amount, reporter gene activation was reconfirmed individually for each prey plasmid and subsequently analyzed in a bait dependency test, where the reporter activation for a prey was compared between BSEP and an unrelated, non-interacting bait. Sequencing and identification of the cDNA fragments by alignment search tools led to elimination of the remaining false positive clones, which occurred due to out of frame readings or gene products from non-relevant compartments.

Secondly, immunoprecipitation of BSEP from human liver and consecutive analysis of the co-precipitated protein complexes by MS/MS allowed for the identification of proteins associated with the transporter. For solubilization of BSEP we tested two commonly used, mild detergents, Triton X-100 and digitonin. To identify BSEP-specific precipitation, protein frequencies from co-IP with BSEP antibody were compared to the frequencies obtained with a control antibody.

The adaptor protein complex 2 (AP-2) is a known interaction partner of BSEP (8) and was precipitated in this setup. Subunits of the complex were detected by MS/MS after solubilization of canalicular membranes with Triton X-100, while specific AP-2 signals were absent in samples prepared with digitonin (Fig. 2, A). Immunoblots of

the samples however showed that subunits of AP-2 were precipitated with BSEP in both detergents (Fig. 2, B).

Thirdly, *in vitro* pull-down analyses were used to confirm a subset of identified interaction partners using recombinant, purified proteins. Here, the alpha subunit of the AP-2 complex as a known interaction partner was able to pull down BSEP (Fig. 3 B).

The subcellular localization of the eleven identified BSEP interaction partners is depicted in Fig. 4. In the following section, we will focus on the proteins that were identified with these complementary methods (Tab. 1).

Factors of the early secretory pathway.

The majority of proteins identified in both screens are membrane proteins of the early secretory pathway. All proteins discussed in the following paragraph co-precipitated specifically with BSEP from human liver and required Triton X-100 for solubilization (data not shown). Also, these proteins showed direct interaction with BSEP as seen in the bait dependency test performed after the MYTH screen (Fig. 5).

The translocon associated protein (TRAP) is a complex of four subunits involved in membrane protein topogenesis (22). The two screens identified the gamma subunit (*SSR3*) as a direct interaction partner of BSEP, while the delta subunit (*SSR4*) was found to associate with the transporter in the co-IP / MS/MS screen.

Another factor connected to the translocation of hydrophobic protein sections is the translocating chain-associated membrane protein 1 (*TRAM1*). TRAM1 was found to be stimulatory or for some targets even necessary for membrane protein translocation. Furthermore, a role for TRAM in dislocation of proteins from the ER has been postulated (23, 24).

Another aspect of trafficking is membrane protein sorting and quality control. Bap31 (*BCAP31*) is an ubiquitous, integral ER membrane protein, which is closely associated with TRAM and involved in protein sorting. Its expression affects a number of membrane proteins, for example CFTR (*ABCC7*) (25). Some mutations involving *BCAP31* could be connected to liver dysfunction and cholestasis (26, 27).

The receptor expression-enhancing proteins (*REEP*) of which proteins 5 and 6 were identified in MYTH and co-IP / MS/MS, respectively, are proposed to be ER-shaping proteins which directly interact with cargo proteins to modulate their processing and trafficking (28).
The immediate early response 3 interacting protein 1 (*IER3IP1*) is a less studied, ER-resident protein (29). Two mutations have been linked to microcephaly and an abnormal amount of apoptosis (30). Its homology to the yeast protein Yos1 points towards a function in vesicular transport between Golgi and ER.
Retention in endoplasmic reticulum 1 (*RER1*) is an early Golgi membrane protein that is thought to function as a sorting chaperone, which modulates the fate of several membrane proteins (31). While RER1 came up in both screens, in the MYTH screen only the C-terminal half of the 196 amino acid protein was encoded on the library plasmid. This part codes for the last transmembrane helix and the cytosolic tail of the protein. Studies of the yeast homolog Rer1 show that cargo recognition occurs via transmembrane domain interaction, while the C-terminus has been shown to bind to the coatomer (32).
There is little information on two further interaction partners identified. Transmembrane protein 14A (*TMEM14A*) was found to localize to or close to ER and mitochondria and ectopic expression resulted in apoptosis suppression (33). Its molecular function has not yet been elucidated. Transmembrane protein 205 (*TMEM205)* has been identified in endosomes of the liver and other secretory tissue and its expression was linked to increased cisplatin resistance (34). Current information would support a role of this small membrane protein in secretion or vesicular trafficking.

All identified proteins are candidates that may directly determine the fate of BSEP at different stages along the secretory pathway.

BSEP interacts with two ER-anchored enzymes

In addition to the factors related to protein sorting and quality control, BSEP showed interaction with two types of ER-anchored enzymes.

The first enzymes belong to the ER-resident UDP-glucuronosyltransferase 1 family. Co-IP / MS/MS and MYTH identified two closely related members of this family,

polypeptide A4 and A5 (*UGT1A4*/*A5*), respectively. UGT1A4 is able to glucuronidate endogenous steroids and xenobiotic compounds and is inhibited by bile salts (35-37). UGT1A5 shows only minimal glucuronidation activity (38). Of the latter protein, only the C-terminal half was encoded on the library plasmid. The catalytic activity of UGTs is attributed to the N-terminal domain, which reaches into the ER lumen (39).

The bile acyl-CoA synthetase (BACS, *SLC27A5*) is an ER-anchored enzyme that catalyzes the first step in re-conjugation of bile acids to taurine or glycine (40). BACS was found to co-precipitate with BSEP as seen in the MS/MS analysis from samples solubilized with Triton X-100 (Fig. 2, A). With the detergent digitonin corresponding peptides were not found in co-IP samples. Immunoblots of the co-IP samples also showed that BACS is precipitated to a greater degree in the presence of BSEP (Fig. 2, B). Interestingly, a relative of BACS, the very long-chain acyl-CoA synthetase (VLACS, *SLC27A2*), was specifically co-precipitated with BSEP in Triton X-100 (Fig. 2 A). BACS was also identified as a BSEP-interacting protein in the MYTH (Fig. 3 A). Here, the C-terminal 50 amino acids were encoded by the library plasmid, a fragment that belongs to the cytosolic part of the enzyme. This fragment shares 50% identity with the related VLACS. Pull-down analyses, as seen in Fig. 5 B, confirm the interaction of BSEP with the cytosolic part of BACS ($BACS_{77\text{-}690}$).

BSEP interacts with the membrane-cytoskeletal cross-linker radixin

Lastly, radixin (*RDX*) was identified as an interaction partner of BSEP. This protein belongs to the ezrin/radixin/moesin (ERM) family and cross-links membrane proteins to the cytoskeleton (41). The N-terminal FERM (4.1/ezrin/radixin/moesin) domain interacts with target membrane proteins, for example MRP2, and is only accessible if the protein is activated or the inhibiting C-terminus removed (10, 42). The C-terminal ERM-associated domain (C-ERMAD) binds the actin cytoskeleton. Alternatively, ERM proteins can bind other scaffolding proteins, such as NHERF1/EBP50 (41). In the MYTH assay (Fig. 3 A) a radixin fragment was produced that roughly corresponds to the N-terminal FERM domain. Looking at the co-IP results, radixin showed differential precipitation in the two detergents used. While there was no specific co-precipitation with Triton X-100, the complex of BSEP and radixin was stable in the presence of the detergent digitonin (Fig. 2, A). Immunoblot analysis of the co-IPs mirrored the results obtained from MS/MS analysis. With digitonin radixin

appears in the presence of BSEP only, while with Triton X-100 it is also present in the control lanes, suggesting nonspecific binding in that detergent (Fig. 2, B). Pull-down analyses, as seen in Fig. 5 B, confirmed the interaction of BSEP with the N-terminal half of radixin (radixin$_{1-318}$). Additionally, we could show that the C-terminal affinity tags used for purification of BSEP do not influence its interaction with radixin. After proteolytic removal of the BSEP affinity tags, pull-down assays show that radixin$_{1-318}$ can bind BSEP, while full-length radixin does not display a signal as depicted in Fig. 5 C.

One likely protein interaction partner for BSEP was not detected in our approaches. The scaffolding protein NHERF1 is an interaction partner of several ABC-transporters in the liver, for example CFTR, MRP2 and MRP4 (12, 43, 44). NHERF1, like radixin, is predominantly found at the apical membrane of hepatocytes (45) and was tested separately with MYTH, co-IP/ MS/MS and *in vitro* pull-down. We did not detect interaction of NHERF1 and BSEP in any of the three methods applied here.

DISCUSSION

Protein-protein interaction is the basis of many cellular processes and plays a pivotal role in the trafficking, localization and activity of membrane proteins. In the case of the bile salt export pump this has been shown for interactions that modulate its cycling at the canalicular membrane (7-9). In the study presented here, we identified eleven novel proteins that interact with BSEP using a three-sided approach.

The first approach was used to screen for proteins that bind directly to BSEP. The MYTH assay probed BSEP interaction with soluble and membrane-associated liver proteins. Of the over 500 initial candidates 37 were verified as interaction partners after applying the appropriate controls (Fig. 6). In contrast to currently available methods for human tissues, the MYTH assay has the advantage of detecting both stable and more transient interactions. The 26 additional BSEP interaction partners detected by MYTH, which were not found by co-IP / MS/MS may represent these short-lived interactions (Supplemental Table 1). On the other hand the host organism, the required fusion proteins and the quality of the cDNA library limit the detection range.

Immunoprecipitation of BSEP from human liver and subsequent identification of interaction partners by MS/MS resulted in more than 500 BSEP-associated proteins (Fig. 6). This approach allowed for identification of physiological interaction partners and proteins stably associated with the transporter via mediators. On the downside, the method is not suitable for reliable detection of transient interactions and the required use of detergent can disrupt or even prevent physiologically relevant interactions. On the one hand we used digitonin for solubilization. By binding cholesterol (46) it readily disrupts cholesterol-rich membranes and was able to preserve the interaction of BSEP and radixin at the plasma membrane. Triton X-100, on the other hand, was required to access interactions in the secretory pathway.

The third method, *in vitro* pull-down, was used to confirm the interaction of BSEP with soluble proteins or soluble domains of identified interaction partners. The method requires soluble or solubilized proteins and detects stable interactions.

Applying the three presented approaches, which covered both *in vitro* methods as well as procedures for detecting physiological interactions in human liver samples, established the validity of the found interactions. Proteins detected by an individual

technique may still be relevant and require a different method for verification. Since methods for detection of PPI directly in human tissue are at present very limited, *in vitro* and yeast-based approaches help to cover the cases in which PPI can not yet be screened and visualized *in vivo*.

The interaction partners identified in the early secretory pathway are likely involved in the topogenesis, sorting and quality control of BSEP. The TRAP complex, of which subunits gamma and delta have been found to associate with BSEP, assists in membrane protein topogenesis. Interaction of the nascent protein with the TRAP complex may stabilize certain orientations (22). Influence on the sorting of secretory proteins has been shown for TRAM (23) and Bap31, for the latter in the sorting of CFTR (25). Members of the REEP family influence the shape of ER membranes and processing of their target proteins (28) and may influence BSEP trafficking by locally altering ER membrane shape. RER1 is highly conserved across species and has been studied in more detail in the yeast (47). In combination with previous studies our findings indicate that Rer1 interacts with BSEP via its C-terminal transmembrane domain. Since several of the interacting proteins in the early secretory pathway are conserved among species, it is possible that yeast enabled an interaction in the MYTH with endogenous factors.

The glucuronosyl transferases UGT1A4 and A5 are highly homologous enzymes, which associated with BSEP in our study. The UGT1A family has identical C-terminal halves, while their N-termini, which contain the catalytically active residues, are dissimilar (48). It is therefore possible that UGTs share a common binding region with BSEP. The direct interaction partner, UGT1A5, showed very low glucuronidation activity towards tested substrates in a study by Finel et al. (38). Several UGTs are able to bind bile acids and are inhibited by them, which leads us to speculate about UGT1A5 functioning as bile acid sensor in the context of BSEP regulation (37).

The second type of enzyme that was found to interact with BSEP were the acyl-CoA synthetases BACS and VLACS. Their similarities, especially in the C-terminal 50 amino acids that were found to bind to BSEP, may point to a common binding site. BACS and VLACS are both catalyzing steps in the synthesis of BSEP substrates and

may be involved in a regulatory feedback loop that modulates trafficking or modification of the bile salt transporter.

Lastly, BSEP interacts with the FERM domain of the cross-linking protein radixin. Radixin has been shown to connect several membrane proteins to the cytoskeleton either directly or via adaptors. In the case of BSEP and MRP2 Wang et al. observed an influence of radixin expression on localization and transport efficiency in rat hepatocytes, where silencing of radixin led to retention of the transporters in subapical compartments (11).

In summary, with the complementary methods of membrane yeast two-hybrid, *in vitro* pull down assay and co-immunoprecipitation from human liver samples combined with MS/MS we screened for novel interaction partners of BSEP and identified eleven proteins. These results define a network of interacting proteins involved in topogenesis, trafficking and functional regulation (Fig. 4) and suggest that BSEP is tightly controlled within the cell with respect to localization and function. In addition to the factors in the early secretory pathway, radixin and the bile acyl-CoA synthetase have a possible role in the regulation of the transporter. How these interaction partners regulate BSEP in the physiological context remains to be investigated. Our work provides the foundation for further research on post-translational BSEP regulation by protein-protein interaction.

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Author names in bold designate shared co-first authorship

FIGURE LEGENDS

Fig. 1 ***Schematic diagram of the membrane yeast two-hybrid system (MYTH) to screen for interaction partners of the ABC-transporter BSEP.***
The MYTH is based on a split-ubiquitin approach. The bait, BSEP, is fused to the C-terminus of ubiquitin (Cub) and a transcription factor (LexA-VP16). The preys are soluble or membrane-associated liver proteins, introduced via a cDNA library. They are fused to the N-terminus of ubiquitin (NubG). Upon interaction of BSEP and the liver protein the ubiquitin moieties, which have a low affinity for each other due to a mutation in the N-terminus, come into close proximity. The reassembled ubiquitin is recognized by endogenous ubiquitin specific proteases (UBP). The transcription factor is cleaved off and activates the reporter genes (*HIS3*, *ADE2*, *lacZ*).

Fig. 2 ***co-IP / MS/MS identifies BSEP interaction partners.***
A. Immunoprecipitation of BSEP coupled to complex mass spectrometry reveals new interaction partners of BSEP in human liver. Crude canalicular membrane preparations were solubilized in either digitonin or Triton X-100, and immunoprecipitated samples were subjected to MS/MS. Protein frequencies from co-IP with BSEP antibody are plotted against the negative control with naïve mouse IgG. Interaction partners of interest are labeled. For the sake of clarity proteins found exclusively in either the BSEP or control co-IP are depicted with an MS-score of one instead of zero on the other axis. **B.** Immunoblot analysis of co-IPs shown in A.

Fig. 3 ***BSEP interaction with radixin and the bile acyl-CoA synthetase.***
A. MYTH bait dependency test of radixin$_{1\text{-}318}$ and BACS$_{640\text{-}690}$ against BSEP or a non-interacting control bait. **B.** Pull-down of BSEP with radixin$_{1\text{-}318}$, BACS$_{77\text{-}690}$ and AP-2 µ1. Protein interaction partners were immobilized as bait on Strep-Tactin Sepharose and purified BSEP was added. BSEP and the interaction partners were detected by immunoblot analysis with monoclonal antibodies against BSEP or the Strep-tag, respectively. Strep-Tactin Sepharose without bait protein served as negative control. **C.** Tag-less BSEP can be pulled down with radixin$_{1\text{-}318}$, but not with full-length, non-activated radixin. Protein interaction partners were immobilized on Strep-Tactin Sepharose. Purified, tag-less BSEP was added to the beads and the complexes were eluted after washing. BSEP and the interacting proteins were detected by

immunoblot analysis with monoclonal antibodies against BSEP or the Strep-tag, respectively.

Fig. 4 ***Schematic overview of the subcellular localization of identified BSEP interaction partners.***
The eleven novel interaction partners of BSEP are shown in a cellular context. Depicted are the nucleus (N), endoplasmic reticulum (ER), Golgi apparatus (Golgi) and cytoskeleton at the apical membrane (Cyts).

Fig. 5 ***Bait dependency test with BSEP interaction partners in the early secretory pathway.***
To confirm the interaction with prey proteins from the initial MYTH screen, reporter gene activation was tested individually for each prey against the bait, BSEP, or a non-interacting control bait, large T antigen. The figure shows one representative result for the positively tested preys, which are denoted by their respective gene name.

Fig. 6 ***Venn diagram of potential BSEP interaction partners obtained with MYTH and co-IP / MS/MS***
The numbers represent the potential BSEP interaction partners identified by MYTH and co-IP / MS/MS and the overlapping set of proteins found in both screens.

FIGURES

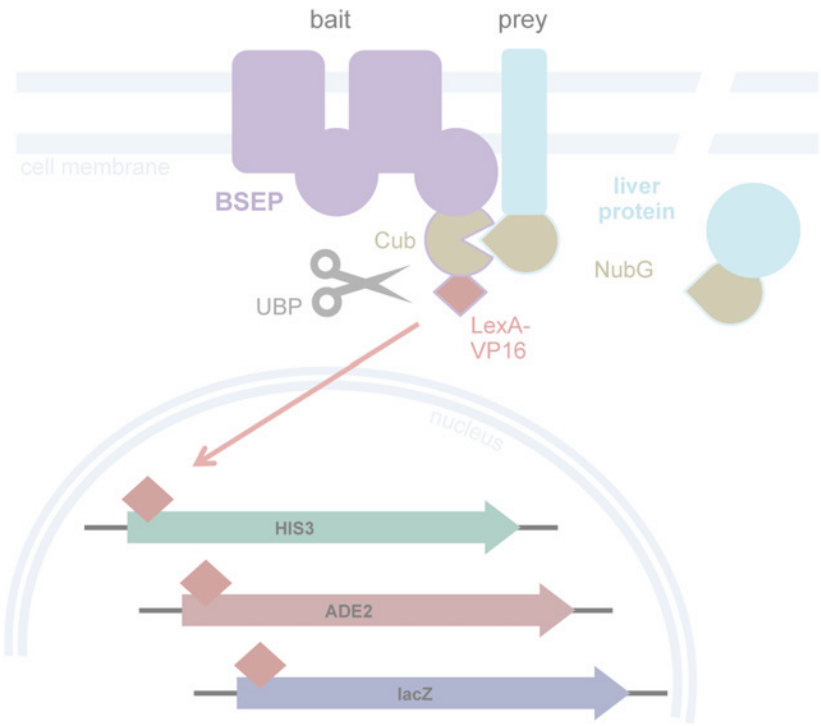
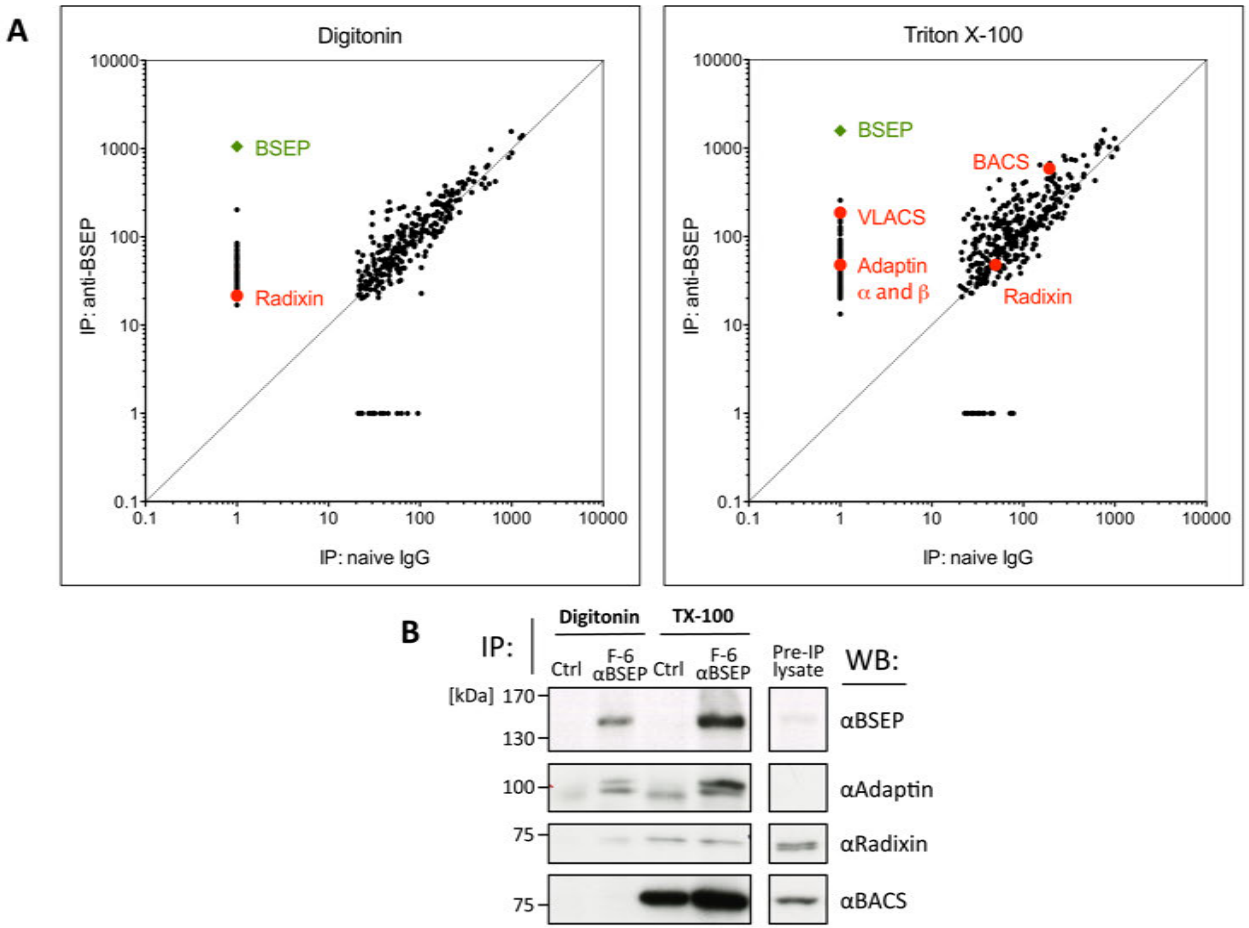


Fig. 1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Triton X-100
BSEP
Radixin
BACS
VLACS
Adaptin
α and β
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IP: naive IgG
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IP:
Digitonin
TX-100
Ctrl
F-6 αBSEP
Pre-IP lysate
WB:
[kDa]
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130
100
75
αBSEP
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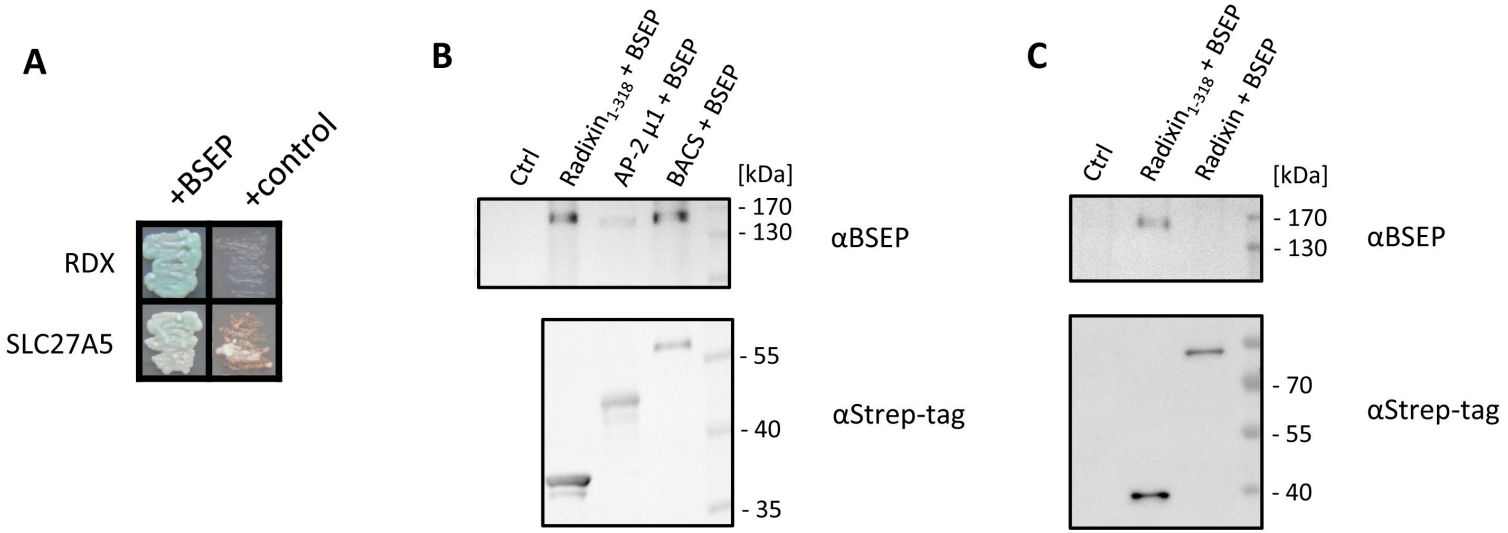
αBACS

Fig. 2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+control
RDX
SLC27A5
B
Ctrl
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AP-2 µ1 + BSEP
BACS + BSEP
[kDa]
- 170
- 130
αBSEP
- 55
- 40
- 35
αStrep-tag
C
Ctrl
Radixin1-318 + BSEP
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- 170
- 130
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- 70
- 55
- 40
αStrep-tag

Fig. 3

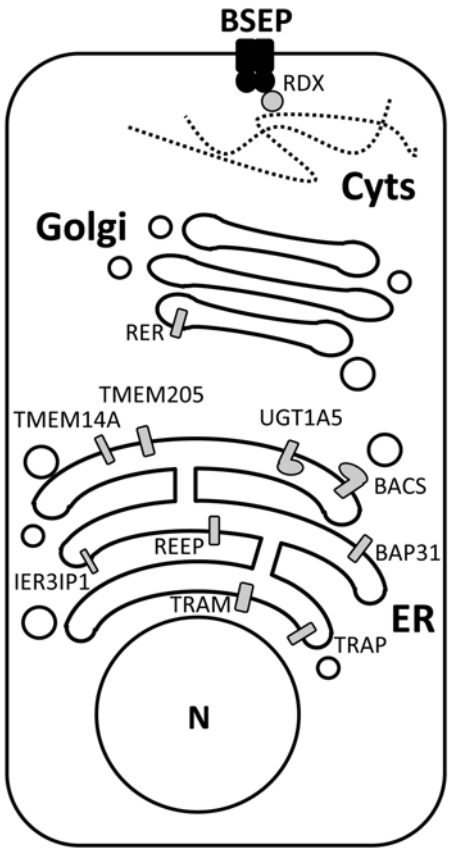


Fig. 1

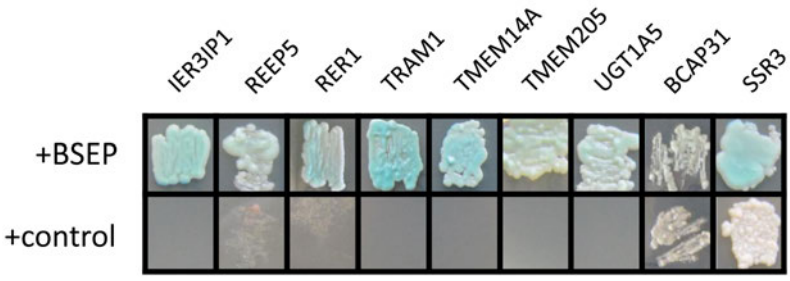


Fig. 2

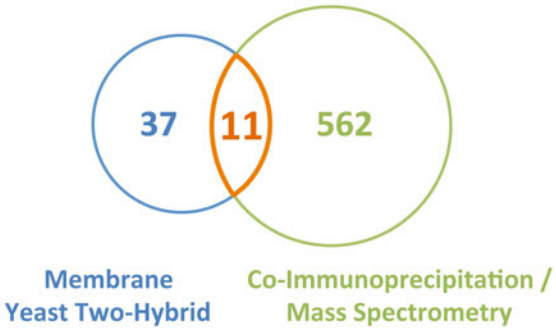


Fig. 3

TABLE LEGEND

Tab. 1 Proteins identified as interaction partners of BSEP in MYTH and co-IP / MS/MS screens. Gene names of closely related proteins identified in the co-IP screen are added in square brackets.

TABLE

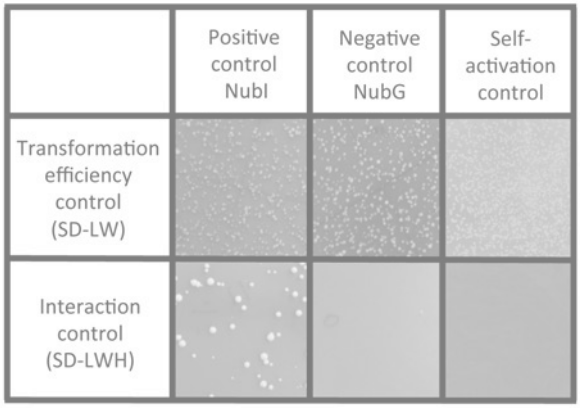
Tab. 1

Protein and Gene Name	Accession Number
B-cell receptor-associated protein 31 (BCAP31)	NM_001256447.1
bile acyl-CoA synthetase (SLC27A5) [SLC27A2]	NM_012254.2
immediate early response 3 interacting protein 1 (IER3IP1)	NM_016097.4
receptor accessory protein 5 (REEP5) [REEP6]	NM_005669.4
RER1 retention in endoplasmic reticulum 1 (RER1)	NM_007033.4
radixin (RDX)	NM_001260492.1
signal sequence receptor, gamma (translocon-associated protein gamma) (SSR3) [SSR4]	NM_007107.3
translocation associated membrane protein 1 (TRAM1)	NM_014294.5
transmembrane protein 14A (TMEM14A)	NM_014051.3
transmembrane protein 205 (TMEM205)	NM_001145416.1
UDP glucuronosyltransferase 1 family, polypeptide A5 (UGT1A5) [UGT1A4]	NM_019078.1

SUPPLEMENTARY

Supplemental Fig. S 1. ***Functional control assay for the membrane yeast two-hybrid with BSEP and self-activation control.***

The yeast strain NMY51 was transformed with the BSEP bait construct and control plasmids coding either for a nonsense peptide with the NubI (wild-type ubiquitin) or the NubG (mutated ubiquitin) -tag. Due to its affinity to its C-terminal half the NubI moiety activates the system regardless of bait interaction. Both controls show equal transformation efficiency on selective media (SD-LW) for the plasmids (upper panels). The positive control shows yeast growth on selective medium (SD-LWH) due to the affinity of the wild-type ubiquitin moieties. This confirms expression of the BSEP fusion protein. Meanwhile, the negative control shows no reporter gene activation based on unspecific interaction of BSEP with the NubG-nonsense peptide. Additionally, self-activation of the MYTH system with BSEP was tested in a library-scale transformation of the empty pPR3-N library vector.



Supplemental Fig. S 1

Supplemental Table 1. ***Protein and gene names of identified interaction partners of BSEP derived from the MYTH assay.***

Highlighted proteins have also been identified in the co-immunoprecipitation / MS/MS screen.

Supplemental Table 1

Protein and Gene Name	Accession Number
aldolase A, fructose-bisphosphate (ALDOA)	NM_184041.2
asialoglycoprotein receptor 2 (ASGR2)	NM_080914.2
B-cell receptor-associated protein 31 (BCAP31)	NM_001256447.1
BCL2/adenovirus E1B 19kDa interacting protein 3-like (BNIP3L)	NM_004331.2
bile acyl-CoA synthetase (SLC27A5)	NM_012254.2
catechol-O-methyltransferase (COMT)	NM_000754.3
CD63 molecule (CD63)	NM_001780.5
CD99 molecule (CD99)	NM_001122898.1
cofilin 1 (non-muscle) (CFL1)	NM_005507.2
dolichyl-phosphate mannosyltransferase polypeptide 2, regulatory subunit (DPM2)	NM_003863.3
ER membrane protein complex subunit 4 (EMC4)	NM_016454.2
glutamate receptor, ionotropic, N-methyl D-aspartate-associated protein 1 (glutamate binding) (GRINA)	NM_001009184.1
heme oxygenase (decycling) 2 (HMOX2)	NM_002134.3
immediate early response 3 interacting protein 1 (IER3IP1)	NM_016097.4
interferon induced transmembrane protein 2 (IFITM2)	NM_006435.2
interferon induced transmembrane protein 3 (IFITM3)	NM_021034.2
mitogen-activated protein kinase binding protein 1 (MAPKBP1)	NM_001265611.1
protein disulfide isomerase family A, member 6 (PDIA6)	NM_005742.2
radixin (RDX)	NM_001260492.1
receptor accessory protein 5 (REEP5)	NM_005669.4
RER1 retention in endoplasmic reticulum 1 homolog (S. cerevisiae) (RER1)	NM_007033.4
signal peptidase complex subunit 1 homolog (S. cerevisiae) (SPCS1)	NM_014041.3
signal peptidase complex subunit 2 homolog (S. cerevisiae) (SPCS2)	NM_014752.2
signal sequence receptor, gamma (translocon-associated protein gamma) (SSR3)	NM_007107.3
sphingomyelin phosphodiesterase 4, neutral membrane (neutral sphingomyelinase-3) (SMPD4)	NM_017951.4
translocation associated membrane protein 1 (TRAM1)	NM_014294.5
transmembrane protein 134 (TMEM134)	NM_025124.2
transmembrane protein 14A (TMEM14A)	NM_014051.3
transmembrane protein 199 (TMEM199)	NM_152464.1
transmembrane protein 205 (TMEM205)	NM_001145416.1
transmembrane protein 230 (TMEM230)	NM_001009925.1
UDP glucuronosyltransferase 1 family, polypeptide A5 (UGT1A5)	NM_019078.1
unconventional SNARE in the ER 1 homolog (S. cerevisiae) (USE1)	NM_018467.3
VAMP (vesicle-associated membrane protein)-associated protein B and C (VAPB)	NR_036633.1
WD repeat domain 83 opposite strand (WDR83OS)	NM_016145.3
Yip1 interacting factor homolog A (S. cerevisiae) (YIF1A)	NM_020470.2
ZMYM6 neighbor (ZMYM6NB)	NM_001195156.1

2.5. Chapter 5 – Recombinant production of human proteins in *E. coli*: Passing a bottleneck towards molecular investigation of BSEP interaction partners

Contribution to this publication: 80 %

- Cloning of interaction partners and variants
- Expression studies in *E. coli* and *S. cerevisiae* and protein purification
- *In vitro* pull-down analysis
- Writing of the manuscript

Published in: (*in preparation*)

Impact Factor:

Recombinant production of human proteins in *E. coli*: Passing a bottleneck towards molecular investigation of BSEP interaction partners

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Keywords

ATP-Binding Cassette Transporters; Recombinant Fusion Proteins; Affinity Purification; Protein Purification; *Escherichia coli*; Protein-protein Interaction; ATP-Binding Cassette Transporters: Regulation;

FOOTNOTE PAGE

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List of Abbreviations

PPI, protein-protein interaction; MYTH, membrane yeast two-hybrid; MS/MS, tandem mass spectrometry; co-IP, co-immunoprecipitation; AP-2, adaptor protein complex 2; BACS, bile acyl-CoA synthetase; BSEP, bile salt export pump (*ABCB11*) MRP2, multidrug resistance-associated protein 2; COMT, catechol-O-methyltransferase; NHERF, Na^+- H^+ exchange regulatory factor; HAX-1, HS-1-associated-protein-X-1; BAAT, bile acid CoA : amino acid N-acyltransferase; FTL, ferritin light chain; HO-2, heme oxygenase 2 (*HMOX2*)

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ABSTRACT

The bile salt export pump (BSEP) is an essential ABC-transporter of the apical hepatocyte membrane that facilitates bile salt dependent bile flow. Functional BSEP expression at the apical membrane requires various steps from synthesis, modification, quality control and trafficking to the tethering. Most of these processes require protein-protein interaction in which for example modifying enzymes or scaffolding proteins bind to BSEP. Our previous study identified a set of BSEP interaction partners. Investigation of these new interaction partners on a molecular level requires a recombinant protein source and often a purification scheme. Here, we present heterologous production of nine human proteins, which have been identified as BSEP interaction partners, in *Escherichia coli.* We additionally confirm the interaction of the catechol-O-methyltransferase with BSEP in *in vitro* pull-down assays.

INTRODUCTION

Protein-protein interaction (PPI) is a prerequisite for virtually all aspects of cellular function. PPI stabilizes or destabilizes proteins, enables or represses enzyme activity, mediates connection to cellular structures and propagates signals (*1-3*). To study the regulation of a protein therefore also means to study its PPIs.

The bile salt export pump (BSEP, *ABCB11*) is an ATP-binding cassette (ABC)-transporter found almost exclusively at the canalicular membrane of hepatocytes. BSEP facilitates the active transport of bile salts, which are the major organic solute of bile, into the canaliculus and constitutes the driving force for bile salt-dependent bile flow (*4*).

Transcriptional and translational regulation mechanisms as well as posttranslational modification of BSEP have been the focus of several studies (*5-7*). On the other hand, the understanding of posttranslational regulation by PPI is still at the beginning. Only a few interaction partners of BSEP are known, which are mainly involved in anterograde trafficking and recycling from the membrane (*8-10*).

Previously, we have identified a number of novel BSEP interaction partners by applying two complementary approaches (*11*). A membrane yeast two hybrid screen screened a liver cDNA library for unknown interaction partners, while co-immunoprecipitation from human liver and pull-down assays confirmed the interactions.

From the identified set of interaction partners only a subset has been investigated to date. To enable *in vitro* molecular analysis of these interaction partners expression protocols for the production in *E. coli* have been established. This way six more human proteins, some of which have not been produced successfully in *E. coli* before, have been made available for consecutive investigation.

MATERIALS AND METHODS

Materials

Chemicals were purchased in analytical quality at Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), VWR (Radnor, PA) or Sigma-Aldrich (Munich, Germany). Detergents were obtained from Anatrace (Maumee, OH).

Strains

Strains of microorganisms used in this study are summarized in Table 1.

Cloning of potential BSEP interaction partners for expression in *E. coli*

cDNA of potential BSEP interaction partners identified in the previously described membrane yeast two hybrid assay (*11*) was cloned into the pET-51b(+) vector (EMD Biosciences, Inc., Darmstadt, Germany) (Table 2). It was amplified by PCR with the addition of ZraI and KpnI restriction endonuclease sites or complementary overhangs with the primers detailed in Table 3 and inserted into pET-51b(+) via these restriction sites or by Gibson Assembly (*12*), respectively. All constructs were verified by DNA sequencing. Full-length cDNA was purchased at Source Bioscience (Nottingham, UK) as listed in Table 4.

Expression of interaction partners in *E. coli*

E. coli was propagated on LB-Agar (10 g/l tryptone/peptone from casein, 5 g/l yeast extract, 5 g/l NaCl, 15 g/l Agar-Agar) at 37°C. Chemically competent cells of *E. coli* were transformed using a protocol derived from Hanahan (*13*).
LB or LBN medium (10 g/l tryptone/peptone from casein, 2 g/l glucose, 29.2 g/l NaCl) supplemented with antibiotics was inoculated with plasmid carrying strains. The main culture was started to an OD_{600} of 0.09 by adding an overnight starter culture. The culture was incubated at 37°C with shaking until an OD_{600} of 0.3-0.8 was reached. Addition of 0.5 mM IPTG induced expression. After 3 to 20 hours cells were harvested (4000g, 20 min, 4°C) and stored at -20°C. For expression in auto-induction medium (*14*) the main culture was induced with a starter culture to an OD_{600} of 0.09 and incubated at 37°C for 2 h, followed by 18 h at 18°C. During induction samples were taken at regular intervals, the cells sedimented (5000g, 2 min) and stored

at -20°C until further analysis by immunoblot or SDS-PAGE. To increase the amount of soluble, heterologously expressed protein, expression conditions were varied. The parameters included expression strain, medium, temperature during induction and length of induction.

Transformation and cultivation of *S. cerevisiae*

S. cerevisiae was transformed according to the protocol by Gietz et al.(*15*). To cultivate *S. cerevisiae* in liquid media, a colony from an agar plate was used to inoculate minimal medium (SD containing 0.34 % (w/v) yeast nitrogen base, 1 % (w/v) ammonium sulfate, 0.137 % (w/v) amino acid mix, 2 % (w/v) glucose, pH 5.6). Expression in the main culture was achieved by changing the carbon source to galactose (SG, identical to SD but with galactose instead of glucose). Cultures were incubated at 30°C and 200 rpm for up to 16 h.

Purification of heterologously expressed proteins

Proteins heterologously expressed in *E. coli* were purified according to the protocol detailed in Przybylla et al. *(11)*. In brief, *E. coli* cells were suspended in about 2 ml of lysis buffer (50 mM Phosphate buffer pH 7, 100 mM NaCl, 1 mM EDTA pH 8, 20 % (w/v) Glycerol, Protease inhibitor) per gram wet cell weight. The cell suspension was then lysed by two passes through a MP-100 Microfluidizer at 1.3 kbar. Unbroken cells and cell debris was removed by differential centrifugation at 2000g, 15 min, 4°C and 14.000g, 30 min, 4°C. The lysate was applied to Strep-Tactin Seapharose for affinity purification, washed and eluted in buffer (50 mM HEPES pH 7, 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin).
For analysis, proteins were separated in discontinuous polyacrylamide gels under denaturing conditions. Immunoblots were probed with the antibodies listed in Table 5.

Immobilized metal ion affinity purification of HAX-1

HAX-1 was additionally cloned into a pET-16b vector, which adds an N-terminal hexahistidine tag to the protein. Protein production and cell lysis was performed as detailed above. HAX-1 was purified by immobilized metal ion affinity purification with a HiTrap Chelating Column (GE Healthcare, Freiburg, Germany) loaded with Ni^{2+}. Protein was washed with IMAC wash buffer (50 mM Tris-HCl pH 8, 150 mM

NaCl, 10 % glycerol) and eluted in IMAC elution buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 10 % glycerol, 300 mM imidazole) and analyzed as described above.

Heterologous expression and purification of BSEP

Recombinant BSEP for pull-down analysis was produced in *Pichia pastoris* and purified as described previously *(11, 16)*.

Pull-down

Pull-down assays for analysis of protein-protein interaction were performed as described previously (*11*). In short, 10 µl of Strep-tactin affinity resin was incubated with heterologously expressed bait fusion protein to saturate binding capacity. Incubation took place for 30 min at 4°C with rotation. After three washing steps (200g, 2 min, 4°C) the resin was incubated with 50 µg of purified BSEP. The complexes were eluted in SDS-sample buffer after five washing steps and analyzed by immunoblot.

RESULTS

In a previous study, we have identified a number of protein interaction partners of BSEP in a combinatorial approach of a Membrane Yeast Two-Hybrid Screen and immunoprecipitation of BSEP and consecutive analysis of complexes by tandem mass-spectrometry. To investigate their interaction with the ABC-transporter *in vitro* a source of significant amounts of protein is required.

Escherichia coli is a convenient and readily available organism that offers fast and high yield protein production. Production of eukaryotic proteins in a prokaryote has some disadvantages, especially a lack of solubility and posttranslational modifications, but few other organisms provide equally simple manipulation of the coding sequence and high yields. Low protein solubility can be addressed by different strains and production conditions.

To produce potential BSEP interaction partners in *E. coli* the coding sequence for each protein was cloned into the pET-51b vector, which adds an N-terminal Strep-tag II to the protein and puts expression under control of a T7 promoter.

In addition to the previously described results a further six proteins were successfully produced in *E. coli* (*11*).

Radixin

Radixin belongs to the Ezrin-Radixin-Moesin (ERM) family of cross-linking proteins. Like the other family members radixin consists of three domains, an N-terminal FERM (band 4.1 and ERM homology)-domain, an intermediary, α-helical domain and a C-terminal actin binding domain called C-ERMAD (for C-terminal ERM-association domain). By binding membrane factors with the FERM domain and the actin cytoskeleton with C-ERMAD radixin helps forming part of the membrane-associated cytoskeleton (*17*). In the inactive state the N- and C-terminal domains are binding each other and therefore inhibit binding of other proteins (*18*). Only after activation, which involves binding of phosphoinositol (4,5)-bisphosphate (PIP_2) and phosphorylation of a threonine residue in the actin binding site, can the two termini bind to their respective partners.

In our previous work we could show that radixin binds to BSEP via its N-terminal FERM domain (*11*). The full length protein as well as the truncated version, which contains the FERM domain (aa 1-318), could be produced in *E: coli* BL21. For improved solubility the expression took place in high-salt medium (LBN) and at low temperature (18°C) (Figure 1).

Following production, radixin and its FERM domain could be purified to 90 % homogeneity in one step (Figure 2), comparable to previously reported purification (*19*).

NHERF1

The Na^+/H^+ exchange regulatory factor 1 (NHERF1), also known as Ezrin-Radixin-Moesin-binding phosphoprotein 50 (EBP50), is another mediator of membrane-cortex interaction. Several membrane proteins have been shown to bind to NHERF1's PDZ domains (*20, 21*). Additionally, as the alternate name suggests, NHERF1 is capable of binding ERM proteins via its ERM binding motif and is therefore an important mediator of membrane-cortex binding (*22*).

NHERF1 was a probable candidate for BSEP interaction and was therefore over-produced for investigation. The methods applied previously did not indicate an interaction with BSEP, though.

Production of NHERF1 in *E. coli* has previously been reported by Park et al. (*23*) in a similar approach. In our study, soluble expression was low at 37°C and variation of expression conditions led to the optimal conditions of induction at 18°C with the addition of 3 % ethanol. Resulting expression and purity after a one-step purification gave similar results to the previously published method, although here, most likely due to the molecular chaperone ethanol and the reduced temperature, the amount of soluble protein was more than ten times higher (Figure 3).

HAX-1

HS-1-associated-protein-X-1 (HAX-1) belongs to a family of ubiquitously expressed proteins. The 35 kDa protein has been shown to interact with a large number of other proteins, among them BSEP (*8*). While an anti-apoptotic effect has been observed during overexpression of HAX-1, its exact physiological role is still unknown (*24*).

Initially, HAX-1 could not be produced in *E. coli* in significant amounts from the pET-51b plasmid. Expression was not detectable or weak and the produced protein was insoluble (data not shown). Variation of expression conditions could not significantly increase the protein amounts. The highest expression of HAX-1 from the pET-51b vector was detected in auto-induction medium with the strain Rosetta (DE3) pLysS (Figure 4), but the amounts were not detectable by Coomassie-staining and too low for further applications.

In the case of HAX-1 baker's yeast was used as an alternate production organism. As an easy to handle eukaryote, yeast is also capable of achieving high protein yields. HAX-1 cDNA was cloned into the galactose inducible pESC-HIS vector. With the tested system yeast did not allow for production of HAX-1 though (data not shown).

The highest soluble expression was achieved in *E. coli* NiCo21 (DE3) from the plasmid pET-16b, which codes for a 6x His-tag fusion peptide. A significant amount of protein was still insoluble, but the remaining protein was sufficient for purification (Figure 5). With the provided system, binding and the yield was yet insufficient to enable further investigation.

AP-2 µ1

The 49.7. kDa subunit µ1 of the adaptor protein complex 2 (AP-2) has been shown to interact with BSEP by Hayashi et al (*9*). The entire AP-2 complex has been crystallized by Kelley et al. and the structure was determined (*25*). In their approach all four subunits of the complex were simultaneously expressed in *E. coli* and the subunits originated from different species, with µ1 subunit from rat. To our knowledge production of the human µ1 subunit in *E. coli* has not been reported yet.

In our approach, the µ1 subunit was produced mostly as insoluble inclusion bodies. Optimal production of µ1 was achieved in *E. coli* strain BL21 (DE3) with an overnight induction at 18°C. The fusion protein had an apparent molecular weight of 53 kDa as seen on SDS-PAGE and immunoblot (Figure 6). Obtained amounts were sufficient for use in pull-down analysis as seen in our previous study (*11*).

Bile Acid Acyl-CoA Synthetase

The bile acid acyl-CoA synthetase (BACS) is an ER-resident, membrane-associated enzyme that catalyzes the first step of bile acid conjugation to glycine or taurine (*26, 27*). BSEP shows the highest affinity for conjugated bile acids and they are the predominant substrate of the transporter (*28*).

As previously reported, BACS was identified by three complementary methods as an interaction partner of BSEP (*11*). Both the full-length, membrane-anchored protein and a soluble variant with the N-terminal transmembrane helix deleted (aa 77-690) were tested for production in different *E. coli* strains. Only the soluble variant was produced in significant amounts as seen in Figure 7. Expression levels for the shorter variant were high, but the protein was mostly insoluble. Only after optimization of expression conditions sufficient amounts of soluble enzyme were produced for pull-down analysis (Figure 7).

Bile Acid CoA: Amino Acid N-Acyltransferase

The bile acid CoA : amino acid N-acyltransferase (BAT/BAAT) is an enzyme involved in bile acid metabolism. BAAT, following BACS, catalyzes the second step of bile acid conjugation to the amino acids glycine or taurine (*29*).

BAAT co-immunoprecipitated together with BSEP from human liver, while the interaction could not be confirmed in the membrane yeast two-hybrid. Therefore expression of the enzyme in *E. coli* was of interest to enable further *in vitro* assays.

BAAT cDNA was cloned into the pET-51b vector in two variants: with an N-terminal or a C-terminal Strep-tag. The N-terminally tagged BAAT could be produced in the

prokaryote, though despite optimization soluble expression levels remained too low for further investigation (Figure 8).

Ferritin Light Chain

Ferritin is an ubiquitously expressed, iron-binding protein complex consisting of light and heavy chains. The light chain, unlike the heavy chain, shows no enzymatic activity towards iron ions (*30*). This 20 kDa chain has been identified in the membrane yeast two-hybrid as an interaction partner of BSEP.

It was possible to express soluble ferritin light chain (FTL) in a straightforward approach in BL21 at 37°C (Figure 9).

Cofilin 1

Cofilin is a 19 kDa protein found ubiquitously in the cytoplasm of cells. It catalyzes the first step of actin polymerization and controls spatial and temporal actin dynamics (*31*).

Cofilin has been identified as an interaction partner of BSEP in a membrane yeast two-hybrid screen. Analysis of co-immunoprecipitation from human liver did not reveal this protein. If the interaction is physiologically relevant this may point to a transient interaction or one that is dependent on the presence or absence of certain posttranslational modifications.

Cofilin could be expressed and purified in high amounts from *E. coli*. Straightforward production for three hours at 37°C yielded large amounts of soluble protein, which could be purified to >90 % homogeneity by affinity purification (Figure 10).

Heme oxygenase 2

Heme oxygenases (HO) are responsible for the degradation of heme into its components biliverdin, CO and free iron (*32*). In the study of a cholestasis model by Donner et al. induction of HO-1, which shows 60 % similarity to HO-2, increased biliary excretion of taurocholate by improved canalicular expression of BSEP *(33)*. In the previous MYTH study HO-2 was identified as an interaction partner of BSEP.

Full-length HO-2 (36 kDa) could be produced in *E. coli*, but the amount of soluble protein was too low for further purification and investigation even after optimization (Figure 11).

Catechol-O-methyltransferase

The catechol-O-methyltransferase (COMT) is a 24 kDa soluble cytosolic protein that catalyzes the O-methylation of catechol neurotransmitters, xenobiotics and metabolites of estrol and estradione (*34*). COMT was another candidate identified for interaction with BSEP by the membrane yeast two-hybrid screen and a consecutive pull-down assay.

COMT was readily produced in *E. coli* under standard conditions and could be purified by affinity chromatography to about 80 % homogeneity (Figure 12).

Interaction of purified COMT with BSEP was investigated by pull-down assays. As seen in Figure 13 immobilized COMT could pull down BSEP. The reverse set-up also showed interaction albeit with a weaker signal (data not shown).

In summary, ten human proteins were successfully produced in *E. coli*, eight of them in sufficient soluble amounts. Table 6 summarizes the optimal conditions determined for the soluble expression of the BSEP interaction partners. Five of the proteins were additionally successfully purified to at least 80 % homogeneity. Our previous study identified BACS, radixin and nine further proteins as BSEP interaction partners. Here, its interactome could be expanded by the catechol-O-methyltransferase.

DISCUSSION

A bottleneck of *in vitro* investigation of protein-protein interaction is the availability of the interaction partners. For BSEP, our group could previously establish an expression and purification protocol, which allowed for heterologous production of the transporter (*16, 35*). In this study, interaction partners of BSEP that were identified in an earlier study by a membrane yeast two-hybrid screen were produced in *E. coli* to enable molecular investigation.

Prokaryotic expression systems have a few disadvantages in regard to recombinant expression of human proteins. Conditions in the host such as pH, codon usage or chaperones can be unfavorable for some heterologous proteins (*36-38*). Also, the lack of posttranslational modifications may hinder further investigation. On the other hand, few expression systems can compete with the speed, inexpensive production and high yield of the prokaryotic expression systems (*37*). In this study, most of the chosen BSEP interaction partners, which were soluble or soluble fragments of human liver proteins, were successfully produced in *E. coli*.

Two known interaction partners of BSEP, HAX-1 and the AP-2 complex, were chosen as possible controls for future interaction assays. HAX-1 proved to be difficult to express in both *E. coli* and *S. cerevisiae*. Ultimately, Coomassie-visible amounts after purification were obtained by expression from a pET-16b plasmid in *E. coli* NiCo21 cells. So far, there is no posttranslational modification of HAX-1 known, which could render *E. coli*-produced protein unstable or inactive (*24*). The human µ1 subunit of AP-2 was more readily produced in *E. coli* enabling the use of this protein as a control as shown in our earlier study (*11*).

NHERF1, which does not directly interact with BSEP (*11*), but may play a role in its connection to the cytoskeleton by binding to radixin, was obtained in high amounts from *E. coli*. We could improve on a similar, previously reported expression of this scaffold protein in *E. coli* (*23*) by lowering the induction temperature to obtain high amounts of soluble protein. NHERF1 is regulated by phosphorylation, which for example promotes its dimerization (*21*). Protein obtained from *E. coli* may therefore

not be fully functional and require a eukaryotic expression host for certain downstream investigations.

Additionally, we show here the expression of the previously investigated bile acyl-CoA synthetase and radixin, as well as the bile acid CoA: amino acid N-acyltransferase. BAAT co-precipitated with BSEP from human liver, but was not identified in the yeast two-hybrid approach in contrast to radixin and BACS. Unfortunately, the enzyme could not be produced in sufficient amounts to facilitate purification and molecular investigation. Falany et al. were able to produce amounts of BAAT in *E. coli* XL-1 that sufficed for radiolabeled activity assays, which the method presented here probably exceeds (*39*).

While the heme oxygenase 2 showed only low soluble amounts, both cofilin 1 and the ferritin light chain were readily overproduced by *E. coli* and are now available in soluble form. Consecutive cofilin investigation will be limited to the actin-binding form of the enzyme, since the inactivated, phosphorylated variant cannot be produced in *E. coli* (*31*). The ferritin light chain has no known posttranslational modification and its potential role in the regulation of protein secretion, as observed for hepatocellular ApoB, can be a starting point for the investigation of its interaction with BSEP (*40*).

Finally, we also overproduced the catechol-O-methyltransferase in the course of our study and confirmed the interaction with BSEP observed in the MYTH by pull-down analysis. As an enzyme that catalyzes methylation reactions with estrogens, which play a role in the long-term regulation of canalicular ABC-transporters (*28, 41*), it will be interesting to further characterize this interaction and its physiological relevance.

With the procedures detailed here, in addition to the previously examined proteins, NHERF1, HAX-1, ferritin, colfilin 1 and the catechol-O-methyltransferase are now available for molecular investigation. Further work can now be directed towards determination of the structural basis and kinetic parameters of the interactions.

ACKNOWLEDGEMENTS

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FIGURES

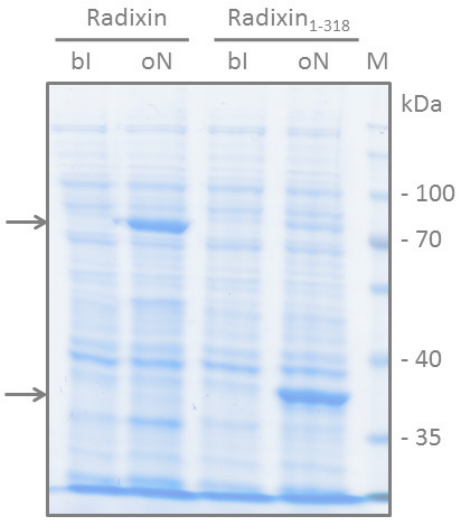

Figure 1 Production of radixin and radixin FERM domain (aa 1-318) in *E. coli*

Radixin and the first 318 amino acids of radixin were produced in *E. coli* BL21 (arrows). Cells were harvested after overnight induction (oN) at 18°C. Samples before induction (bI) were taken as control. Protein size standard was applied for reference (M).

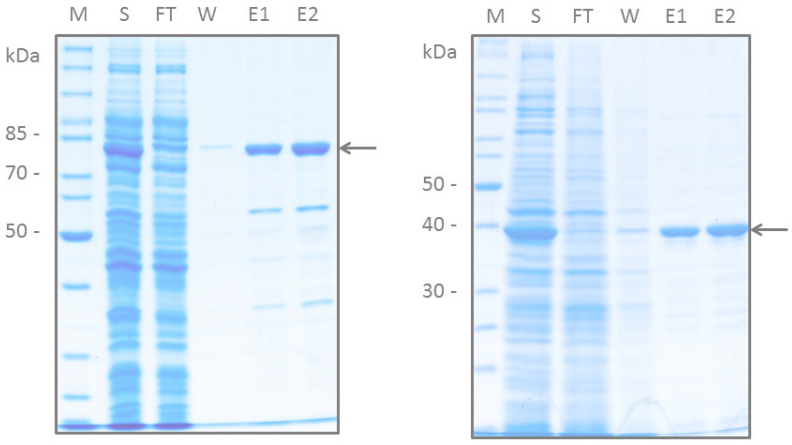

Figure 2 Affinity purification of radixin and radixin FERM domain

Radixin (left) and the N-terminus of radixin (right) have been purified via Strep-tactin affinity purification to >90 % homogeneity. After application of the cell lysate supernatant (S) to the column and collection of the flow through (FT), the column was washed (W) and bound protein eluted (E1-2).

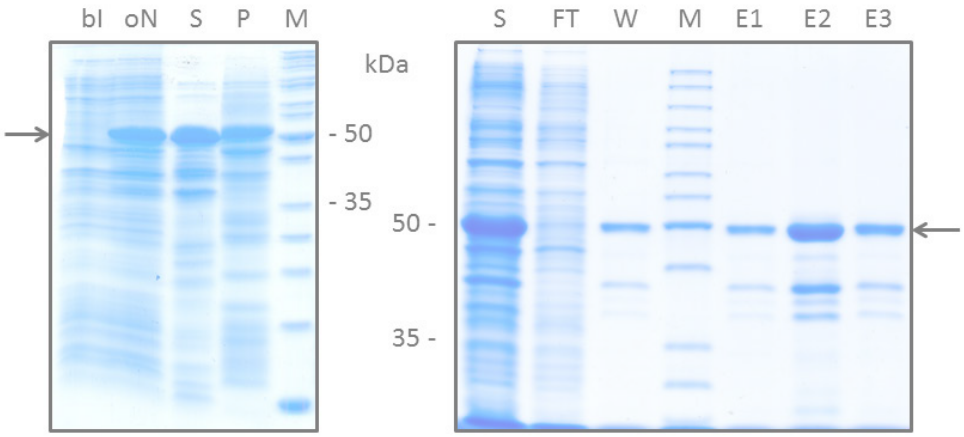

Figure 3 Production and affinity purification of NHERF1

NHERF1 was produced in *E. coli* BL21 (arrows). Cells were harvested after overnight induction (oN) at 18°C. Samples before induction (bI) were taken as control and soluble protein (S) separated from insoluble inclusion bodies (P). Protein size standard was applied for reference (M). NHERF1 could be purified via Strep-tactin affinity purification to >85 % homogeneity. After application of the cell lysate supernatant (S) to the column and collection of the flow through (FT), the column was washed (W) and bound protein eluted (E1-3).

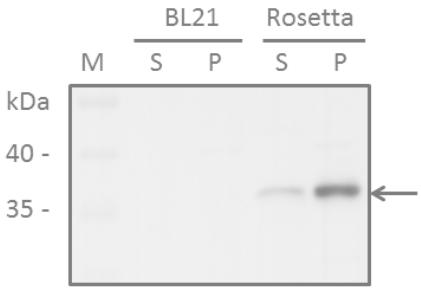

Figure 4 Production of HAX1 in *E. coli*

Soluble HAX1 was produced in *E. coli* Rosetta (DE3) pLysS (arrow). Cells were harvested after overnight incubation at 18°C in auto-induction medium. Amounts of soluble protein (S) were compared to insoluble protein (P). Protein size standard was applied for reference (M).

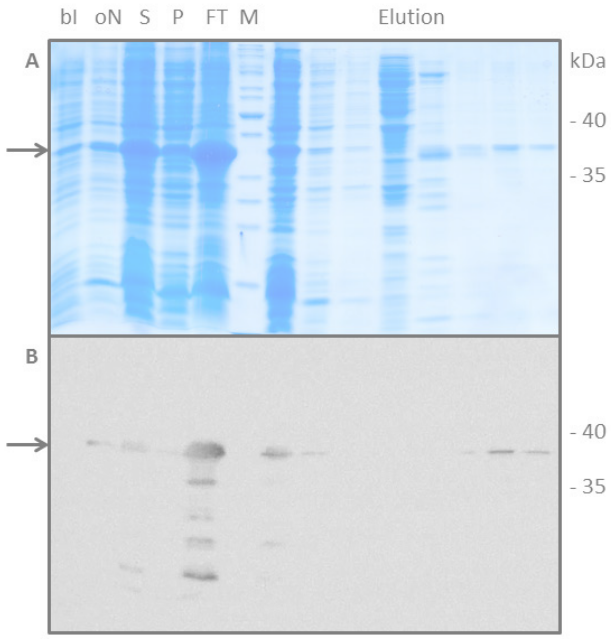

Figure 5 Production and affinity purification of HAX1

HAX1 was expressed from the pET16b vector in the NiCo21 strain at 30°C with addition of 3 % ethanol to the medium (oN). After cell lysis insoluble proteins (P) were sedimented and the supernatant (S) was purified by immobilized metal ion affinity chromatography. Unbound protein was collected in the flow through (FT) and bound protein eluted with an imidazole gradient. **A** Coomassie stained SDS gel and **B** immunoblot developed with the PentaHis antibody.

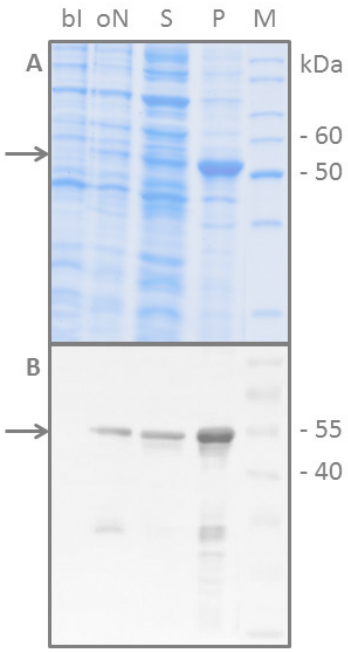

Figure 6 Production of AP-2 µ1 in *E. coli*

The µ1 subunit of the AP-2 complex was produced in *E. coli* BL21 (DE3) (arrows). Cells samples were harvested before induction (bI) and after overnight incubation at 18°C (oN). Amounts of soluble protein (S) were compared to insoluble protein (P). Production was analyzed by **A** SDS-PAGE and by **B** immunoblot with antibody against the Strep-tag. Protein size standard was applied for reference (M).

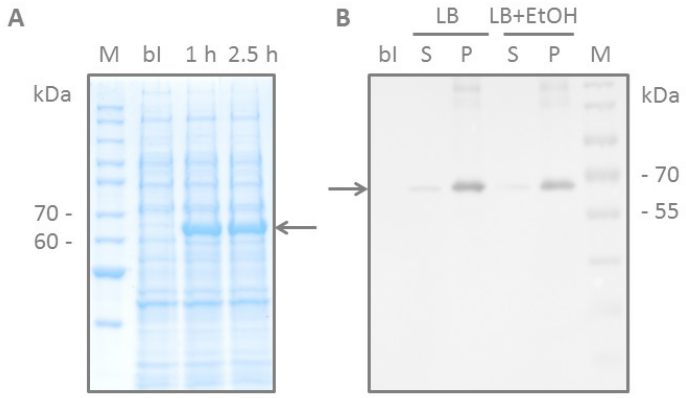

Figure 7 Production of BACS in *E. coli*

The bile acyl-CoA synthetase was produced in *E. coli* Rosetta (DE3) pLysS (arrows). **A** Cells samples were harvested before induction (bI) and after one and 2.5 hours after induction at 37°C and analyzed by SDS-PAGE. **B** Only after induction at 18°C and analysis by immunoblot with anti-Strep antibody was a significant amount of soluble protein (S) detected compared to insoluble protein (P). Protein size standard was applied for reference (M).

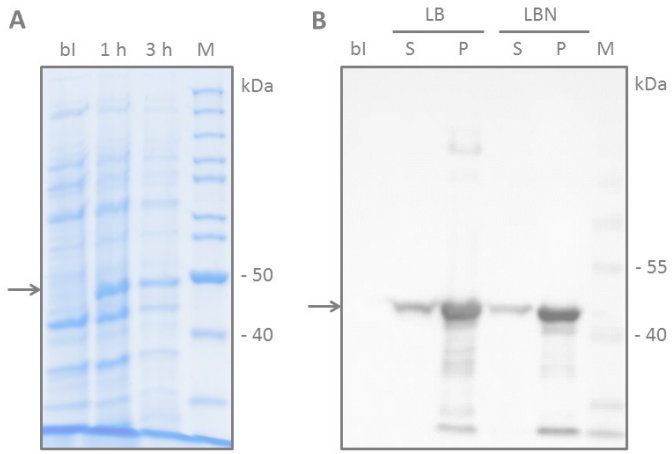

Figure 8 Production of BAAT in *E. coli*

The bile acid CoA : amino acid N-acyltransferase (BAAT) was produced in *E. coli* BL21 (DE3) (arrows). **A** Cells samples were harvested before induction (bI) and after one and 3 hours after induction at 37°C and analyzed by SDS-PAGE. **B** Variation of expression conditions, such as the medium (LB, LBN), yielded small amounts of soluble protein (S) as compared to insoluble protein (P) detected by immunoblot with antibody against the Strep-tag. Protein size standard was applied for reference (M).

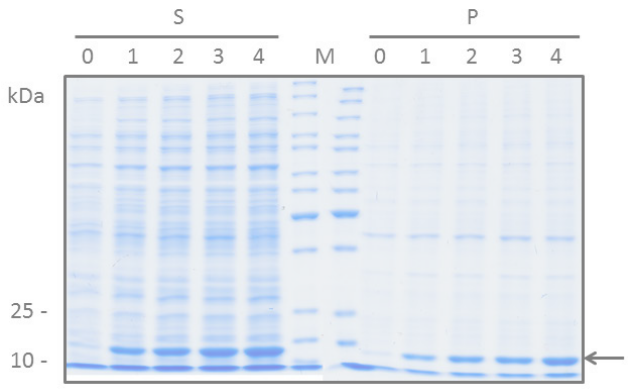

Figure 9 Production of ferritin light chain

A Ferritin was produced in *E. coli* BL21 (DE3) (arrows). Cells samples were harvested before induction (0) and up to four hours after induction at 37°C (1-4) and analyzed by SDS-PAGE. Amounts of soluble protein (S) was compared to inclusion body production (P). Protein size standard was applied for reference (M).

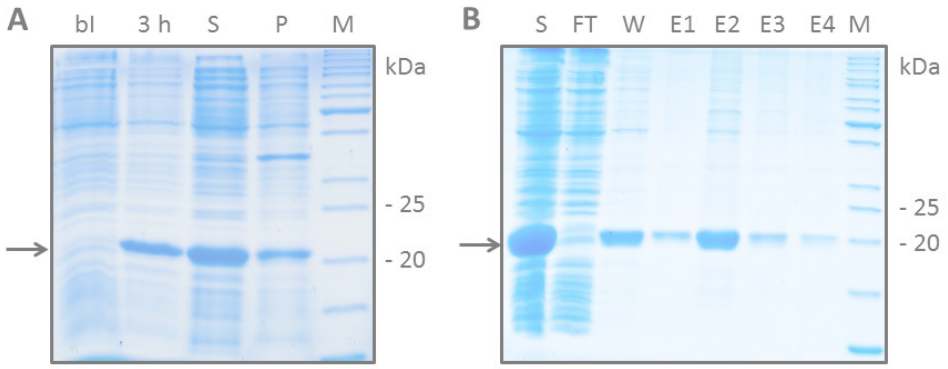

Figure 10 Production and purification of cofilin

A Cofilin was produced in *E. coli* BL21 (DE3) (arrows). Cells samples were harvested before induction (bI) and after three hours induction at 37°C and analyzed by SDS-PAGE. Amounts of soluble protein (S) was compared to inclusion body production (P). **B** Cofilin was purified by Strep-tactin affinity purification. Cleared lysate (S) was applied to the column and the flow through (FT) collected. After washing (W) bound protein was eluted (E1-4). Protein size standard was applied for reference (M).

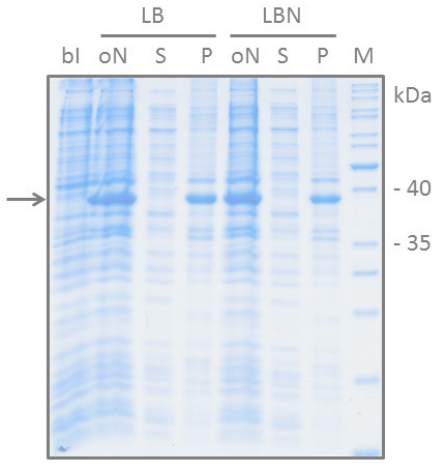

Figure 11 Production of heme oxygenase 2

A HO-2 was produced in *E. coli* BL21 (DE3) (arrow). Cells samples were harvested before induction (bI) and after overnight induction at 18°C (oN) and analyzed by SDS-PAGE. Amounts of soluble protein (S) was compared to inclusion body production (P). Protein size standard was applied for reference (M).

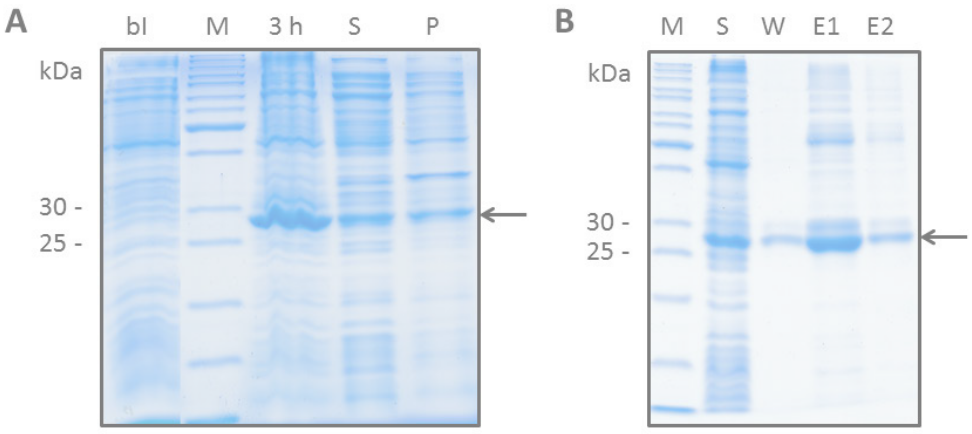

Figure 12 Production and purification of catechol-O-methyltransferase

A Catechol-O-methyltransferase (COMT) was produced in *E. coli* BL21 (DE3) (arrows). Cells samples were harvested before induction (bI) and after three hours induction at 37°C and analyzed by SDS-PAGE. Amounts of soluble protein (S) was compared to inclusion body production (P). **B** COMT was purified by Strep-tactin affinity purification. Cleared lysate (S) was applied to the column. After washing (W) bound protein was eluted (E1-2). Protein size standard was applied for reference (M).

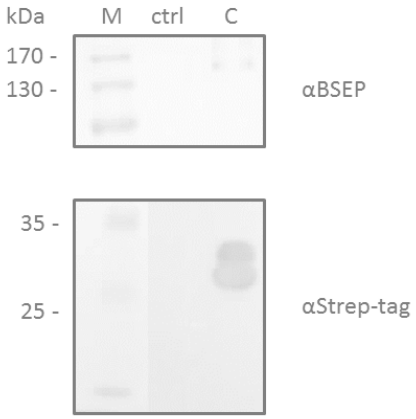

Figure 13 Pull-down of BSEP with catechol-O-methyltransferase

Purified catechol-O-methyltransferase (C) was immobilized for pull-down analysis and incubated with purified BSEP. Protein complexes were eluted after washing and analyzed by immunoblot with antibodies against BSEP and Strep-tag. Free affinity resin served as control. Protein size standard was applied for reference (M).

TABLES

Table 1 Strains of microorganisms used in this study

Name	Genotype	Source
	E. coli	
BL21 (DE3)	*fhu*A2 [*lon*] *omp*T *gal* (λ DE3) [*dcm*] Δ*hsd*S λ DE3 = λ *sBamHIo* Δ*EcoRI-B int*::(*lac*I*::*P*lac*UV5::T7 *gene*1) *i21* Δ*nin5*	NEB (Ipswich, MA)
BL21 (DE3)pLysS	*E. coli* B F$^-$ *dcm omp*T *hsd*S(r_B^- m_B^-) *gal* λ(DE3) [pLysS Cam^r]	Agilent (Santa Clara, CA)
Rosetta (DE3)pLysS	F$^-$ *omp*T *hsd*S$_B$(r_B^- m_B^-) *gal dcm* (DE3) pRARE2 (Cam^R)	Novagen
NiCo21 (DE3)	*can*::*CBD fhu*A2 [*lon*] *omp*T *gal* (λ DE3) [*dcm*] *arn*A::*CBD sly*D::*CBD glm*S6*Ala* Δ*hsd*S λ DE3 = λ *sBamHIo* Δ*EcoRI-B int*::(*lac*I::P*lac*UV5::*T7 gene*1) *i21* Δ*nin5*	NEB
DH5α	F$^-$ φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ$^-$	Invitrogen
XL1-blue	*rec*A1 *end*A1 *gyr*A96 *thi-1 hsd*R17 *sup*E44 *rel*A1 *lac* [F´ *pro*AB *lac*I^{q}ZΔM15 Tn10(Tet^r)]	Agilent
	S. cerevisiae	
YPH500	*MAT*α *ura3-52 lys2-801_amber ade2-101_ochre trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1*	Sikorski and Hieter, 1989

Table 2 Plasmids constructed or used in the course of this study

Name	ORF	Ref
pET-51b-RDX	radixin	Przybylla (*11*)
pET-51b-RDX-N	radixin$_{1-318}$	Przybylla (*11*)
pET-51b-NHERF1	NHERF1	this study
pET-51b-HAX1	HAX1	this study
pET16b-HAX1	HAX1	this study
pESC-HIS-HAX1	HAX1	this study
pET-51b-CFL1	Cofilin1	this study
pET-51b-COMT	catechol-O-methyltransferase	this study
pET-51b-HMOX2	heme oxygenase 2	this study
pET-51b-BACScom	bile acyl CoA-synthetase	this study
pET-51b-BACSsol	bile acyl CoA-synthetase $_{77-690}$	Przybylla (*11*)
pET-51b-FTL	FTL	this study
pET-51b-AP2M1	AP-2 mu subunit 1	this study
pET-51b-BAAT	BAAT	this study

Table 3 Oligonucleotides used in this study

Name	Sequence 5'-3'
pESC-M2_for	AGAAGGACCCGGGGCCTGGCAAGCTGGAG
pESC-M2_rev	GCCGCAAGCTCGAGGACCGACGTCGGATC
18-2-CFL1_for	ACG ACA AGG TAC CAA TGG CCT CCG GTG TG
18-2-CFL1_rev	CGA TGA TGA GCT CTT ATC ACA AAG GCT TGC CC
58-2-COMT_for	GAT CGA TGA CGT CAA TGG GTG ACA CCA AGG
58-2-COMT_rev	CGA TGA TGA GCT CTT ACG GGG CAG TCA GGG
28C-2-HMOX2_for	ACG ACA AGG TAC CAA TGT CAG CGG AAG TGG
28C-2-HMOX2_rev	GAT CGA TGA CGT CGC TTC ACA TGT AGT ACC AGG
delRDX-C_for	TGA GAG CTG TTA TTT TGC ATA TAT G
delRDX-C_rev	TTA CAA CTG CTT CTG ATG TTT CTC
delRDX-CwoS_rev	CAA CTG CTT CTG ATG TTT CTC
BACSsol_for	ACG ACA AGG TAC CAA TGC CAG CAC GGC TG
BACScom_for	ACG ACA AGG TAC CAA TGG GTG TCA GGC AAC AG
BACS_rev	GAT CGA TGA CGT CTT AGA GCC TCC AGG TTC C
205A-FTL_for	ACG ACA AGG TAC CAA TGA GCT CCC AGA TTC GTC AG
205A-FTL_rev	GAT CGA TGA CGT CTT AGT CGT GCT TGA GAG TGA G
petAP2M1_for	ACG ACA AGG TAC CAA TGA TTG GAG GCT TAT TCA TC
petAP2M1_rev	GAT CGA TGA CGT CCT AGC AGC GAG TTT CAT AAA TG
BAAT-Gib_for	GTA TCA ACG CAG AGT GGC CAT TAT GAT CCA GTT GAC AGC TAC CC
BAAT-Gib_rev	GAT ATC GAA TTC TCG AGA GGC CGT TAG AGT TGA CTG GTC ACA TCT GG
pBR3	TCCCCATCGGTGATGTC
T7-981079	TAATACGACTCACTATAG

All oligonucleotides were purchased at MWG Biotech (Ebersberg, Germany).

Table 4 Purchased plasmids and their properties

Name	ORF	Reference
pOTB7-AP2M1	adaptor protein complex 2 (AP-2) mu 1 subunit	IRAU19C02
pENTR223.1-SLC27A5	SLC27A5 (bile acyl CoA-synthetase, BACS)	OCAB14E06
pDNR-LIB-BAAT	bile acid CoA : amino acid N-acyltransferase (BAT/BAAT)	IRAUp969E0551D

Table 5 Antibodies used in this study

Antibody	Source
anti-Mouse HRP goat monoclonal	Dianova, Hamburg, Germany
anti-Strep tag II mouse monoclonal	Millipore, Darmstadt, Germany
PentaHis mouse monoclonal	Qiagen, Hilden, Germany

Table 6 Optimized production conditions for potential BSEP interaction partners in *E. coli*

Protein	Strain	Medium	Induction at	Inductor	Temperature
Radixin	BL21 (DE3)	LBN	OD 0.5	0.5 mM IPTG	18°C üN
Radixin$_{1-318}$	BL21 (DE3)	LBN	OD 0.5	0.5 mM IPTG	18°C üN
NHERF1	BL21 (DE3)	LB	OD 0.5, +3% EtOH	0.2 mM IPTG	18°C üN
HAX1	BL21 (DE3)	ZYM-5052	-	-	2 h 37°C, 18°C üN
HAX1 (pET16b)	NiCo21 (DE3)	LB	OD 0.5 +3 % EtOH	0.5 mM IPTG	4 h 30°C,
AP-2 µ1	BL21 (DE3)	LB	OD 0.5	0.5 mM IPTG	18°C üN
BACS$_{77-690}$	Rosetta (DE3) pLysS	LB	OD 0.3	0.5 mM IPTG	18°C üN
FTL	BL21 (DE3)	LB	OD 0.5	0.5 mM IPTG	3 h, 37°C
Cofilin	BL21 (DE3)	LB	OD 0.5	0.5 mM IPTG	3 h, 37°C
COMT	BL21 (DE3)	LB	OD 0.5	0.5 mM IPTG	3 h, 37°C

3. DISCUSSION

The liver's many secretory functions require the translocation of a variety of compounds across the cell membranes. Catalysis of these processes depends on active transporters. Particularly ABC-transporters in the canalicular hepatocyte membrane are integral to the secretion of bile and xenobiotics.

Two liver ABC-transporters are in the focus of this thesis: firstly, the bile salt export pump, BSEP. Dysfunction as in hereditary trafficking defects of this transporter result in impaired bile flow, which increases incidence of fibrosis, cancer and liver failure (63). Secondly, the multidrug transporter ABCG2 participates in the resistance of cancer cells against chemotherapeutics and with its wide substrate spectrum also influences pathogenesis of other diseases such as gout and Alzheimer's (31).

Major aims of this thesis were to improve the availability of the human ABC-transporters for molecular investigation by establishing and optimizing recombinant expression systems and purification schemes and to identify PPI partners with a potential regulatory function.

3.1. A Fast, Recombinant Expression System Yields Active ABCG2

A prerequisite for a number of methods in *in vitro* analysis is a source for considerable amounts of pure protein. Physiological protein levels in the host are usually not sufficient for these purposes. Therefore, recombinant protein production is often the method of choice. Many overexpression systems have been developed and improved over the last decades. ABCG2 function has first been investigated in the human cancer cell lines, in which it was first identified (27, 29). Other well-established cell lines and cells such as HEK293 (64), MDCKII (65) and *Xenopus laevis* oocytes (66) soon followed. These systems have the advantage of near physiological conditions concerning posttranslational modifications and membrane composition, but they lack the high yield required for purification and many *in vitro* approaches. Recombinant baculovirus-infected insect cells, e.g. Sf9 (*Spodoptera frugiperda*) (67) and High Five (*Trichoplusia nii*), offered yields, which enabled first structural characterization of ABCG2 by electron microscopy (68). Yeasts were used as one of the most easy to cultivate and inexpensive eukaryotes (69, 70). Preparations from *Pichia pastoris* in particular were used for the determination of transport kinetics and further structural approaches based on 2D crystals analyzed by cryo-electron microscopy (71). One heterologous bacterial expression system was established in *Lactococcus lactis* (72).

Escherichia coli, one of the most well-established model organisms, could not yet be harnessed for recombinant expression of ABCG2. To ease production of the transporter for *in vitro* approaches, that demand high amounts of protein, expression was established in the fast-growing, inexpensive prokaryote *E. coli*. Expression of a codon-optimized ABCG2 gene from a plasmid with T7 promoter was optimized in shaking cultures. ABCG2 was successfully produced as 65 kDa non-glycosylated protein. The molecular weight was comparable to eukaryotic expression systems like insect cells and yeasts, which produce an under-glycosylated protein (67, 69, 70). As previously observed by Pozza et al. (73), although not with the same severity, production of ABCG2 inhibits *E. coli* cell growth, which decreases overall protein yield per liter culture in comparison to less demanding heterologous proteins. *E. coli* is an advantageous host in this case, since up-scaling is straightforward and

fermentation would be a fast and inexpensive alternative process. One disadvantage was the increased degradation observed in the heterologous host. Various attempts at stabilizing the protein by decreasing the induction temperature and addition of molecular chaperones were moderately successful. The amount of degradation products still exceeded 20 % of total ABCG2. The degradation products made the purification process more challenging, but it was still possible to obtain >1 mg of purified ABCG2 per liter culture, which is enough to produce an amount suitable for many types of molecular investigation. As previously observed by others, ABCG2 is resistant to solubilization by most detergents (68, 70). The same proved true for solubilization of ABCG2 from *E. coli* membranes where only Fos-choline 16 could solubilize more than 50 % of the transporter. In the following, the transporter was purified in one step to more than 85 % homogeneity. Other heterologous expression systems, for example insect cells or yeast, showed a similar degree of purity (68, 74, 75). Lastly, correct folding of ABCG2 was verified by its enzymatic ATPase activity. With the addition of cholesteryl hemisuccinate during solubilization ABCG2 stability was improved and an ATP hydrolysis rate of 40 nmol P_i per mg and min determined which is equal to other reported detergent-solubilized transporter preparations (74, 76).

Fast-growing eukaryotic organisms like yeasts have the advantage of posttranslational modifications, glycosylation in particular, which may stabilize ABCG2 (38). On the other hand, the majority of previously reported recombinant expression systems produce under-glycosylated ABCG2 (67, 69, 70). *E. coli* has the advantage of higher protein yields per cell weight and a faster and low-cost expression. Furthermore, the glycosylation is not essential for the transporter's activity (37).

The activity and likely also the stability of ABCG2 depend on the presence of cholesterol in the membrane (77, 78). *E. coli* membranes do not contain cholesterol, which may be a factor resulting in the protein degradation observed during expression. For further *in vitro* investigation reconstitution of ABCG2 into cholesterol-containing membranes could improve stability and activity of the transporter. Expression of ABCG2 in yeasts would not be an alternative, since the prevalent sterol in the membranes, ergosterol (79), may even decrease ABCG2 activity (78).

3.2. BSEP: Protein Interactions From Translation To The Membrane

Protein-protein interaction influences virtually all cellular processes. Particularly the regulation of membrane protein trafficking and activity requires multiple PPIs, an aspect which is difficult to study in *in vivo* systems. With the help of fluorescent fusion proteins or immunolabeling it is possible to assess co-localization, while determination of direct interaction is more complex. To screen the interactome of a protein of interest genetic methods such as yeast two-hybrid approaches can be applied. Other procedures include *in vitro* purification schemes such as tandem affinity purification, co-immunoprecipitation (co-IP) or chemical cross-linking techniques combined with mass spectrometry (62, 80, 81).

Earlier studies identified few BSEP interaction partners with yeast two-hybrid approaches using a soluble BSEP fragment, GST pull-down and co-IP (82-84). The three proteins, HAX-1, the AP-2 complex and myosin II regulatory light chain, are modulators of BSEP cycling and influence abundance of the transporter in the canalicular membrane. The present thesis aimed to explore the interactome of BSEP in more detail with three complementary approaches.

Protein interaction partners of BSEP were identified by a membrane yeast two-hybrid screen (MYTH). The system was established for full-length BSEP and screened human liver proteins encoded by a cDNA library. After application of the appropriate controls 39 novel BSEP interaction partners were identified with the MYTH. Verification of the interactions was approached in a cooperation, where eleven PPIs were validated in the physiological context by co-immunoprecipitation from human liver samples combined with tandem mass spectrometry. A second line of validation were *in vitro* pull-down analyses with purified proteins. Heterologous expression of BSEP has previously been established in the methylotrophic yeast *Pichia pastoris* (85, 86). In the present thesis expression and purification protocols were optimized (Figure 6) and interaction of purified BSEP with soluble interaction partners was tested in pull-down assays (Chapters 2.3, 2.5.).

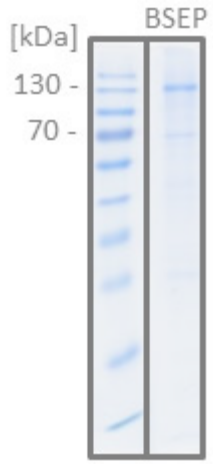

Figure 6 SDS-PAGE of purified BSEP stained with colloidal Coomassie.

BSEP was expressed as a ~150 kDa protein in Pichia pastoris and purified according to the methods in 2.3.

By using an organ-specific cDNA library the MYTH allows for the screening of a tissue's proteome for interaction with a protein of interest. The MYTH assay is tailored to examine both soluble and membrane proteins and has the advantage of detecting stable but also more transient interactions (87). It is limited by the required fusion proteins, which may interfere with an interaction, and false positive hits due to bringing together non-physiological pairs and the different modifications and membrane conditions of the heterologous host. Furthermore, interactions with a protein complex, such as the AP-2 complex, cannot be detected with this technique. Consequently, any results in PPI determination needs to be confirmed with an independent method. The applied co-immunoprecipitation explored the physiological situation, where stable interactions were detected in human liver samples. Furthermore, pull-down analyses were used in this work to confirm BSEP interaction with soluble proteins or soluble domains. A disadvantage of these latter methods is that they are unsuitable to detect transient contacts, which may be the basis for several of the interactions identified in the MYTH. To establish the validity of the remaining interactions identified in the MYTH other methods will be required. More transient interactions may be accessed by chemical cross-linking techniques combined with mass spectrometry (81, 88) or protein-fragment complementation assays such as TOXCAT and bimolecular fluorescence complementation (89, 90).

During the investigation of BSEP interaction partners recombinant expression of several human proteins was established in *E. coli* (2.5). For nine of the interaction partners expression and purification protocols were optimized and >90 % pure protein was obtained for molecular investigation. Some candidates could not be obtained in sufficient amounts, outlining some of the limitations of the prokaryotic host. Many of the identified BSEP interaction partners are membrane proteins. Their expression and purification is more challenging and will require alternative expression systems and optimization until they can be accessed for *in vitro* analysis.

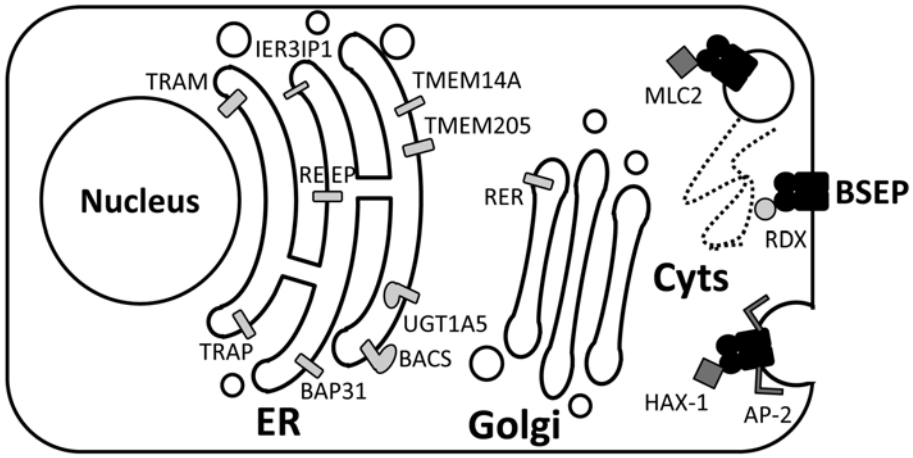

Figure 7 Schematic representation of known BSEP interaction partners in a cellular context

Depicted are the eleven interaction partners of BSEP (black) identified in the course of this thesis in the ER, Golgi apparatus and at the apical membrane with the cytoskeleton (Cyts) (light grey). Additionally, previously known interaction partners during anterograde trafficking and endocytosis are shown in dark grey.

The identified interactions of BSEP trace the path of the transporter from the synthesis at the ER membrane through trafficking along the secretory pathway to the plasma membrane, where BSEP is cycled and tethered to the cytoskeleton (Figure 7). These proteins are candidates, which may participate in the pathogenesis of cholestasis by influencing BSEP topogenesis, trafficking, quality control and tethering. More than hundred BSEP mutations and single nucleotide polymorphisms are known as a cause for cholestasis (61). Some are nonsense mutations, insertions or deletions, while other cause splicing defects or abolish activity (91, 92). Another group of mutations results in reduced expression of BSEP at the plasma membrane (93, 94). How an altered interaction of BSEP with other proteins can influence it's

posttranslational regulation is poorly understood. The newly identified interaction partners are a first step towards a more detailed understanding of BSEP trafficking and regulation.

Future work needs on the one hand include the investigation and verification of further interaction partners, as well as the detailed analysis of the identified interactions. On the other hand the physiological relevance and influence of the interactions needs to be investigated in appropriate *in vitro* and *in vivo* systems.

This thesis laid the basis for future *in vitro* investigation of the liver ABC-transporters ABCG2 and BSEP by establishing and optimizing heterologous expression systems and identification and recombinant production of novel protein interaction partners of BSEP.

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Protein structure figures were prepared with PyMol (http://www.pymol.org).

Chemical structures were taken from PubChem (http://pubchem.ncbi.nlm.nih.gov/)

5. ABBREVIATIONS

ABC	ATP-binding cassette
ATP	adenosine-5´-triphosphate
BS	bile salt
BSEP	bile salt export pump (*ABCB11*)
co-IP	co-immunoprecipitation
d	days
E. coli	*Escherichia coli*
e.g.	exempli gratia
ER	endoplasmic reticulum
g	gram
h	hour
IMAC	immobilized metal ion affinity chromatography
IPTG	isopropyl-β-D-thiogalactopyranoside
L	liter
M	molar (g/mol)
MDR	multidrug resistance
MDR3	multidrug resistance protein 3 (*ABCB4*)
min	minute
MS/MS	tandem mass spectrometry
MYTH	membrane yeast two-hybrid
NBD	nucleotide binding domain
OD	optical density
P. pastoris	*Komagataella pastoris* (previously: *Pichia pastoris*)
P_i	free phosphate
PPI	protein-protein interaction
PTM	posttranslational modification
s	second
S. cerevisiae	*Saccharomyces cerevisiae*
TMD	transmembrane domain
v/v	volume per volume
w/v	weight per volume

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III. EIDESSTATTLICHE ERKLÄRUNG

Ich versichere an Eides Statt, dass die Dissertation von mir selbstständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis“ an der Heinrich-Heine-Universität Düsseldorf erstellt worden ist. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf, 11. Januar 2016 Susanne Przybylla