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Biosynthesis and Trafficking of the Bile Salt Export Pump, BSEP: Therapeutic Implications of BSEP Mutations

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Abstract

The bile salt export pump (BSEP, *ABCB11*) is the primary transporter of bile acids from the hepatocyte to the biliary system. This rate-limiting step in bile formation is essential to the formation of bile salt dependent bile flow, the enterohepatic circulation of bile acids, and the digestion of dietary fats. Mutations in *BSEP* are associated with cholestatic diseases such as progressive familial intrahepatic cholestasis type 2 (PFIC2), benign recurrent intrahepatic cholestasis type 2 (BRIC2), drug-induced cholestasis, and intrahepatic cholestasis of pregnancy. Development of clinical therapies for these conditions necessitates a clear understanding of the cell biology of biosynthesis, trafficking, and transcriptional and translational regulation of BSEP. This chapter will focus on the molecular and cell biological aspects of this critical hepatic membrane transporter.

Keywords

Bile salt secretion; ATP-binding cassette transporter; Cholestasis

1. Introduction

Bile is a largely (~95%) aqueous fluid that is produced by the hepatocyte in the liver and released into the biliary system of ducts and, if present, the gallbladder. It is a complex mixture of endogenous solid constituents, including bile salts, bilirubin, phospholipids, cholesterol, amino acids, steroids, enzymes, porphyrins, vitamins, and heavy metals, as well as exogenous drugs, xenobiotics and environmental toxins. After ingestion of a meal, bile, in the form of mixed micelles of bile salts and phospholipids, gets released into the duodenum where its detergent properties act to solubilize lipids, thus aiding in their absorption by the intestine. Bile salts also stimulate intestinal immunity and function as ligands for G-coupled protein receptors to regulate thermogenesis (Keitel et al., 2008). The rate limiting step in the secretion of bile salts by the liver is achieved by an ABC transporter known as the bile salt

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export pump (BSEP, *ABCB11*). This member of the ATP-binding cassette (ABC) family of proteins is localized to the hepatocyte canalicular membrane and is critical to the formation of bile salt dependent bile flow and the normal enterohepatic circulation of bile salts from the distal intestine back to the liver. Thus, defects in the biosynthesis or trafficking to the apical plasma membrane due to mutations in the gene result in a deficiency in bile flow that is known as cholestasis. *BSEP* mutations have been associated with progressive familial intrahepatic cholestasis type 2 (PFIC2) (Strautnieks et al., 1998), benign recurrent intrahepatic cholestasis type 2 (BRIC2) (van Mil et al., 2004), drug-induced cholestasis (Modica et al., 2009), hormone-dependent intrahepatic cholestasis of pregnancy (ICP), biliary lithiasis (Davit-Spraul et al., 2010) and transient neonatal cholestasis (Hermeziu et al., 2006). This pivotal role in liver function in normal and disease conditions has made it essential to understand the regulation of BSEP in order to develop therapies directed toward treating cholestasis. This review will focus on the cell biology of the synthesis and trafficking of BSEP/Bsep, and how this information is being utilized in treating patients suffering from different forms of cholestasis related to *BSEP*.

2. Structure and function of BSEP

An ATP dependent bile salt transporter was initially described in 1991 by Nishida et al in canalicular membrane vesicles (Nishida et al., 1991) and rapidly confirmed by 3 other groups (Adachi et al., 1991; Muller et al., 1991; Stieger et al., 1992). It was first partially cloned from a pig liver cDNA library based on its similarity to the multidrug resistance protein, P-glycoprotein (MDR1, *ABCB1*) (Childs et al., 1995). Therefore, it was originally named sister of P-glycoprotein (spgp). The full-length coding region was identified in 1998 by Gerloff et al (Gerloff et al., 1998) from rat liver and characterized as the bile salt export pump, Bsep. BSEP consists of 1321 amino acids in humans and has a molecular mass of ~160 kDa. The gene is located to chromosome 2q24-31 in humans (Strautnieks et al., 1997) and mouse *Bsep* is located in a region of chromosome 2 syntenic to 2q24-31 (Lecureur et al., 2000). Rodent Bseps are ~80% identical to human BSEP, whereas Bsep in lower vertebrates such as the marine skate (*Leucoraja erinacea*) has 69% identity to human BSEP (Cai et al., 2001). The crystal structure of BSEP/Bsep has not yet been determined, but as a member of the ABC superfamily, it is predicted to have two segments of six transmembrane domains (TMD) separated by an ~350 amino acid cytoplasmic nucleotide binding domain (NBD) that contains Walker A and B signatures. A molecular model of the structure of BSEP/Bsep has been proposed based on the crystal structure of the multidrug transporter from S. aureus, Sav1866 (Kubitz et al., 2012). Both the N- and C-terminal portions of the molecule are located to the cytoplasmic face of the plasma membrane. The first extracellular loop contains four N-glycosylation sites which act in the stability, trafficking and function of BSEP (Mochizuki et al., 2007). Additionally, BSEP/Bsep can be modified by phosphorylation and ubiquitinylation (Hayashi and Sugiyama, 2009; Kubitz et al., 2004; Noe et al., 2001; Wang et al., 2008).

BSEP/Bsep is responsible for bile salt dependent bile flow. Transport of bile salts is dependent on ATP hydrolysis. It cannot be stimulated by any other nucleotide and is not driven by ATP-dependent pH gradient or an inside positive membrane potential (Adachi et al., 1991; Muller et al., 1991; Stieger et al., 1992). BSEP/Bsep transports primarily

monovalent bile salt species, including taurine and glycine conjugates of primary bile salts, cholic acid (CA) and chenodeoxycholic acid (CDCA), and the secondary bile salt, deoxycholic acid (DCA), as well as ursodeoxycholic acid (UDCA) (Stieger, 2011). Substrate specificity does not vary greatly among species, although differences in the native bile acid pool in different species are likely responsible for the variability (Byrne et al., 2002; Stieger, 2011). The order of affinity for conjugated bile salts for human and rodent BSEP/Bsep is taurochenodeoxycholic acid (TCDCA)= taurocholic acid (TCA)>taurodeoxycholic acid (TDCA)>tauroursodeoxycholic acid (TUDCA)~glycocholic acid (GCA) (Byrne et al., 2002; Gerloff et al., 1998; Green et al., 2000; Noe et al., 2002).

BSEP also has low affinity for certain drugs that are also substrates for MDR1, but pravastatin is the only non-bile salt solute that has been confirmed as a substrate for BSEP (Hirano et al., 2005). However, in vitro studies with human and rodent BSEP/Bsep-expressing cells have shown that some drugs, notably cyclosporine, rifamycin and glibenclamide, can competitively inhibit BSEP/Bsep function (see review (Stieger, 2011) and (Byrne et al., 2002)). It is still unclear whether these drugs directly cause liver injury in humans through their inhibition of BSEP, or whether another “hit”, such as a susceptibility mutation in *BSEP*, predisposes to these drug induced liver diseases (Morgan et al., 2010). See further discussion in Section 5 below.

3. Plasma Membrane Localization of BSEP/Bsep

3.1 Constitutive Expression

BSEP/Bsep is almost exclusively expressed in the liver on the hepatocyte apical, canalicular membrane, evenly distributed throughout the lobular domains. Although there are reports of low levels of mRNA expression in non-hepatic tissue, including the kidney of the sea lamprey (Cai et al., 2012), the protein has never been detected in these tissues (Stieger, 2011). Immunolabeling experiments have shown that Bsep is predominantly expressed on the canalicular plasma membrane on the microvillar, but not the intermicrovillar, membrane (Gerloff et al., 1998). Smaller amounts of the protein are localized to subapical vesicles (Dombrowski et al., 2006; Gerloff et al., 1998). This partitioning of the membrane is believed to reflect lipid microdomains, or rafts, which are enriched in caveolin-1, cholesterol, and sphingomyelin. These domains are resistant to extraction by Lubrol WX (Ismair et al., 2009) and bile salts (Guyot and Stieger, 2011), thus protecting the canalicular membrane from the high concentration of secreted bile salts. Furthermore, these cholesterol rich microdomains are important for the activity of Bsep (Paulusma et al., 2009) and over-expression of caveolin in mice increases bile salt secretion (Moreno et al., 2003).

It is still unclear how BSEP/Bsep traffics to the apical membrane after its synthesis and post-translational modification in the Golgi. Biosynthetic labeling of newly synthesized rat Bsep suggests that it traffics out of the Golgi directly into a subapical, vesicular compartment where it can reside for several hours before moving to the apical plasma membrane (Kipp and Arias, 2000). This subapical compartment consists of rab11-positive endosomes (Wakabayashi et al., 2004). Interestingly, other apical ABC transport proteins, multidrug resistance protein 1 and 2 (Mdr1, Mdr2), also appear to traffic directly to the canalicular membrane from the Golgi, but they do not linger in a subapical vesicular compartment

(Kipp and Arias, 2000; Sai et al., 1999). This direct trafficking is in contrast to apical membrane ectoenzymes, such as dipeptidyl peptidase IV, aminopepdidase N, and the cell adhesion molecule, cCAM105, which first traffic from the ER and Golgi to the sinusoidal membrane before being transcytosed to the apical membrane. However, we have shown that Bsep can reside in the same intracellular vesicle as the transcytotic marker, pIgARec (Soroka et al., 1999). Furthermore, when alloantibodies to BSEP found in some children with PFIC2 after liver transplantation were incubated with isolated rat hepatocytes they were found at the apical, unexposed, plasma membrane within 30 minutes, presumably by binding at the basolateral membrane and trafficking to the apical membrane (Keitel et al., 2009).

The half-life of BSEP/Bsep in the apical membrane is ~4-6 days and the protein is believed to constitutively recycle between the plasma membrane and subapical vesicles (endosomes) containing Rab11 and requiring myosin Vb (Lam et al., 2007; Wakabayashi et al., 2005). In WIF-B9 cells expressing BSEP-YFP this constitutive recycling was microtubule dependent and sensitive to actin inhibitors, but unaffected by brefeldin A, cAMP, TCA, or phosphatidylinositol 3-kinase inhibitors (Wakabayashi et al., 2004). This pathway has been proposed to provide a rapid response for canalicular transporters when bile acid secretion is needed (Kipp et al., 2001). Although this study suggests that BSEP/Bsep transporters are constantly cycling on and off the apical plasma membrane, other research has identified additional proteins that associate with BSEP/Bsep and aid in the “anchoring” of the transporter at it’s site of action. The motor protein myosin II regulatory light chain (MLC2) has been shown to interact with the NBD region of BSEP and to be important in BSEP trafficking to the plasma membrane (Chan et al., 2005). Expression of a dominant negative, non-phosphorylatable form of MLC2 reduced BSEP levels in the apical membrane of MDCK cells and treatment with the MLC2 inhibitor, Blebbistatin, reduced delivery of BSEP to the apical plasma membrane (Chan et al., 2005). Furthermore, Kruglov et al found that the type II inositol 1,4,5 trisphosphate receptor (InsP3R2) colocalizes with Bsep at the canalicular membrane, and loss of this Ca^{++} regulator results in endocytosis of Bsep into an intracellular, pericanalicular compartment with a resultant decrease in bile salt secretion (Kruglov et al., 2011).

The NBD region has also been shown to bind to the cytoskeletal-associated protein, HCLS1-associated protein X-1 (Hax-1), which also interacts with cortactin (Ortiz et al., 2004). Depletion of Hax-1 or over-expression of the dominant negative forms of cortactin or EPS15 leads to the increased level of BSEP in the apical plasma membrane of MDCK cells, suggesting that these proteins are important in internalization of BSEP from the plasma membrane. EPS15 specifically interacts with epsin (Chen et al., 1998) and the AP-2 adaptor (Benmerah et al., 1995), both important in clathrin-dependent endocytosis (Benmerah et al., 1998; Chen et al., 1998). More recently we have shown that the C-terminal tail of the Bsep molecule contains a tyrosine motif (^{1310}YYKLV^{1314}) that is critical to the constitutive endocytosis of Bsep. This motif is conserved among closely related members of the ABCB subfamily of proteins that mediate ATP-dependent transport of substrates of broad substrate specificity. At the same time Hayashi et al (Hayashi et al., 2012) showed that the C-terminal tyrosine motif in BSEP directly interacts with AP-2, an adaptor protein associated with dynamin- and clathrin- dependent endocytosis. Thus, all these data indicate that endocytosis of BSEP/Bsep from the plasma membrane is controlled by its interactions with complexes of

cytoskeletal-associated proteins and suggest that plasma membrane localization, and therefore function, may be regulated through these interactions. Figure 1 summarizes these interactions.

3.2 Regulated Expression

The ability to regulate the amount of BSEP/Bsep in the hepatocyte canalicular membrane is a critical step in controlling the function of BSEP/Bsep in health and disease. Cyclic AMP, TUDCA, and TCA all stimulate insertion of Bsep into the canalicular membrane (Stieger, 2011). Ursodeoxycholic acid has been used in humans to treat cholestasis and its taurine conjugate, TUDCA, has been shown in experimental animal models of cholestasis to stimulate insertion of Mrp2 (*Abcc2*) and Bsep transporters into the apical plasma membrane (Beuers et al., 2001; Dombrowski et al., 2006; Kurz et al., 2001; Micheline et al., 2002). Subsequent studies have shown that p38 MAP kinase and the protein kinase C (PKC) signal transduction pathways are important in this stimulated exocytosis of Bsep by TUDCA from a vesicular compartment to the plasma membrane (Kubitz et al., 2004; Kurz et al., 2001). Hypotonic swelling of cells has also been shown to result in stimulated bile acid excretion and probably acts through activation of Erk-1/2 and p38 MAP kinase (Haussinger et al., 2000; Noe et al., 1996; Schmitt et al., 2001). A phosphoinositide 3-kinase (PI3K) pathway has been shown to be involved in regulated exocytosis of ATP-dependent canalicular transporters by taurocholate, although BSEP was not identified at that time (Misra et al., 1998). Cyclic AMP stimulated secretion of bile salts in isolated perfused rat livers is blocked by microtubule inhibitors, indicating the need for an intact cytoskeleton in this regulation (Hayakawa et al., 1990). Rapid microtubular dependent targeting of Bsep and other canalicular proteins is lost in liver kinase B1 (LKB1) null mice (Woods et al., 2011). LKB1 is an upstream kinase that is activated by bile acids and facilities the establishment of apical polarity in the hepatocyte (Fu et al., 2010; Fu et al., 2011). The LKB1/AMPK pathway is postulated to act at AMPK sites on the plus end of microtubules.

In contrast, induction of cholestasis in rodents by numerous agents including estradiol-17β-glucuronide, lithocholate, LPS and hypoxia result in internalization of Bsep into subapical membrane vesicles and loss of staining of the protein at the canalicular membrane (Crocenzi et al., 2003a; Crocenzi et al., 2003b; Elferink et al., 2004; Fouassier et al., 2007; Micheline et al., 2002; Zinchuk et al., 2005). BSEP is also internalized in cholestatic liver disease in humans (Elferink et al., 2004; Roma et al., 2008). Although the mechanism of regulation in these cholestatic models remains somewhat unclear, multiple pathways have been implicated. Taurolithocholate was shown to act on Bsep membrane localization through protein kinase C epsilon, in a PI3K-dependent manner (Beuers et al., 2003). In addition, lithocholate has been shown to be an antagonist of FXR, thus perhaps also acting through down-regulation of BSEP gene expression (Yu et al., 2002). Estradiol 17-glucuronide induces cholestasis through signaling pathways that include the Ca^{++}-dependent protein kinase C (cPKC) and PI3K (Boaglio et al., 2010; Crocenzi et al., 2008). Sepsis associated cholestasis is mediated by lipopolysaccharide (LPS) and has been shown to act through cytokines, such as Il-6, IL-1α and TNFα (Hartmann et al., 2002; Siewert et al., 2004).

4. Transcriptional regulation of BSEP expression

The level of human BSEP expression varies significantly between individuals (Ho et al., 2010; Tirona, 2011) and its expression is highly regulated by transcriptional mechanisms. The main transcriptional regulator of *BSEP/Bsep* is the nuclear receptor, fanesoid X receptor (FXR, *NH1H4*)(Makishima et al., 1999; Trauner and Boyer, 2003). FXR/Fxr transactivates the proximal promoter of *BSEP/Bsep* in humans (Ananthanarayanan et al., 2001) and in rodents (Gerloff et al., 2002). Bile acids are the physiologic ligand for FXR and thus can regulate the transcription of their own transporter (Makishima et al., 1999). Chenodeoxycholic acid (CDCA) is the major endogenous physiological ligand for FXR (Wang et al., 1999). The critical role of Fxr in Bsep expression is demonstrated by its low basal expression in *Fxr*−/− mice and the lack of an induced response to bile salts (Marschall et al., 2006). This mechanism, together with FXR mediated down-regulation of bile salt synthesis and hepatic uptake transporters, assures that hepatic levels of bile salts should not reach toxic levels. Even in cholestatic liver, BSEP/Bsep expression is relatively sustained (Geier et al., 2007; Lee et al., 2001). Because of this homeostatic mechanism, FXR is a major target for nuclear receptor regulation of cholestatic liver disease (Boyer, 2005; Cai and Boyer, 2006; Stedman et al., 2006). A potent FXR ligand, obeticholic acid, has ~ 100 times affinity for FXR than CDCA and is now in phase lll clinical trials for the treatment of primary biliary cirrhosis.

FXR regulates the expression of target genes by acting together as a heterodimer with retinoid X receptor, RXRα (Forman et al., 1995; Mangelsdorf and Evans, 1995). The human *FXR* promoter also contains receptors for retinoic acid and vitamin D. Activation of the vitamin D receptor with vitamin D3 suppresses FXR activation in in-vitro luciferase reporter assays (Honjo et al., 2006). In addition to FXR, the *BSEP* promoter is also induced by the hepatocyte-specific liver receptor homologue-1 (LRH-*1*, *NR5A2*) (Song et al., 2008). The *BSEP* promoter is positively regulated by the nuclear erythroid 2 p45-regulated factor 2 (NRF2) that plays a significant role in responses to oxidative stress. NRF2 binds to response elements that regulate many hepatic phase I and II enzymes and efflux transporters such as MRP3 and MRP4 (Weerachayaphorn et al., 2009).

5. BSEP Mutations and Disease

The sequencing of the human genome led to the discovery that mutations in *BSEP* caused a form of progressive familial intrahepatic cholestasis in infancy known as PFIC2. This discovery provided the final confirmation that BSEP was indeed the determinant of bile salt dependent bile flow. As additional mutations and polymorphisms in *BSEP* were discovered, it became apparent that genetic mutations in *BSEP* can result in altered BSEP function and subsequent liver disease. These mutations result in a range of mild to severe, progressive forms of intrahepatic cholestasis known as the BSEP deficiency syndrome (Lam et al., 2006; Pauli-Magnus et al., 2005). Patients with PFIC2 mutations are also at risk for hepatocellular carcinoma (Davit-Spraul et al., 2010; Knisely et al., 2006; Strautnieks et al., 2008). A few patients with PFIC2 who have received liver transplants have developed antibody mediated recurrent disease when the newly synthesized BSEP protein is recognized as foreign by the recipient (Jara et al., 2009; Keitel et al., 2009; Maggiore et al., 2010; Siebold et al., 2010).

Common mutations can be grouped into missense mutations, nonsense mutations, deletions and insertions and splice site mutations. In one study of 109 families of patients, most of the mutations were shown to result in decreased or absent BSEP expression on the canalicular plasma membrane (Strautnieks et al., 2008). Another study analyzed 20 mutations/single nucleotide polymorphisms (SNPs) that resulted in reduced wild-type splicing and levels of mRNA in vitro (Byrne et al., 2009). All these mutations can result in truncated or misfolded BSEP proteins that would be subject to quality control mechanisms in the endoplasmic reticulum (ER), thus preventing their expression on the plasma membrane. The most common mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) in patients with cystic fibrosis, Δ*F508*, is a well known example where an ABC transporter is subjected to defective protein folding and processing in the ER (Denning et al., 1992b; Du et al., 2005; Thomas et al., 1992). When seven PFIC2 missense mutations were expressed in MDCK cells, five of these common mutations (G238V, E297G, G982R, R1153C and R1268Q) were unable to traffic to the apical membrane (Wang et al., 2002). Of these, four also lacked the ability to transport taurocholate when expressed in Sf9 cells, again suggesting misfolding and retention by the ER quality control. Two common mutations in human *BSEP*, E297G and D482G, have been reported to have both reduced (Noe et al., 2005; Wang et al., 2002) and normal (Hayashi et al., 2005; Lam et al., 2007) transport activity.

The D482G mutation has also been found to have enhanced aberrant mRNA splicing, perhaps providing an explanation for the variable expression, function and severity of disease (Byrne et al., 2009). This mutation (D482G) is the most common PFIC missense mutation in the European population and 16% of BSEP deficiency patients who develop malignancy have this mutation (Knisely et al., 2006; Strautnieks et al., 2008). Aberrant mRNA splicing in this mutation would result in a truncated protein that would not be able to be detected in immunological assays, thus possibly explaining the lack of detectable BSEP at the canalicular membrane. In addition, Bryne et al have reported that the D482G-containing mRNA is unstable (Byrne et al., 2009). The variability in the clinical phenotype of the E297G mutation, on the other hand, may be due to the ability of the mRNA to be stabilized by splicing factors present in the hepatocyte (Byrne et al., 2009).

Some *BSEP* mutations are associated more frequently with a particular form of cholestasis, suggesting a possible correlation with disease severity (see(Byrne et al., 2009) for a review). We examined differences in protein maturation, plasma membrane localization and transport activity in mutants of rat *Bsep* representing two PFIC2 (D482G and E297G), two BRIC2 (A570T and R1050C) and one ICP (N591) mutation (Lam et al., 2007). It was found that all but the PFIC2 mutation, E297G, retained the ability to transport taurocholate. The reduction in plasma membrane expression correlated equally with reduction in the total amount of Bsep protein, suggesting a defect in protein stability rather than in trafficking. In general, the membrane Bsep expression ranked in the order ICP>BRIC2>PFIC2 (Lam et al., 2007). A similar study also found differences between BRIC2 and PFIC2 in Bsep localization and function after transfection into the polarized cell line, MDCKII (Kagawa et al., 2008).

Young patients with severe BSEP deficiency syndrome are at risk to subsequently develop hepatocellular carcinoma (HCC) and cholangiocarcinoma (Knisely et al., 2006; Scheimann

et al., 2007; Strautnieks et al., 2008), with the reported cases of HCC being much greater than cholangiocarcinoma. These patients frequently present with splice site changes, deletion, insertions, and nonsense mutations that result in the absence of functional protein. This absence results in elevated levels of intracellular bile salts that have been shown to influence many aspects of cell function, including mitochondrial function, cell cycle, DNA repair, homeobox gene activation, and cell polarization and differentiation (Ng et al., 2000; Palmeira and Rolo, 2004; Sokol et al., 2006; Souza et al., 2008; Woolbright and Jaeschke, 2012). Recently it has been shown in HCC patients that the decrease in BSEP expression is associated with an alteration in FXR isoform expression induced by inflammation (Chen et al., 2013). The FXRα2 isoform has more potent activity than FXRα1. In HCC, the ratio of FXRα1/FXRα2 was altered due to the absence of the FXRα2 isoform in one-third of the tumors. The cytokines Il-6 and TNFα increased FXRα1/FXRα2 ratios in Huh7 cells with a subsequent reduction in BSEP expression. These findings raise the possibility that suppression of inflammation in the liver in HCC patients might increase BSEP expression and re-establish bile acid homeostasis.

Some forms of drug induced liver injury (DILI) can be attributed to inhibition of BSEP (Dawson et al., 2012; Fattinger et al., 2001; Pauli-Magnus and Meier, 2006). Animal models of drug-induced cholestasis indicate that cholestatic drugs can inhibit bile secretion and bile acid transport at many levels, including uptake and efflux across the sinusoidal membrane, as well as canalicular efflux. For example, rifampicin, cyclosporine A, rifamycin SV, bosentan, troglitazone, erythromycin estolate, and glibenclamide have all been shown to inhibit Bsep in rats in a dose-dependent fashion (Fattinger et al., 2001; Funk et al., 2001a; Funk et al., 2001b; Stieger et al., 2000). Sulindac also competitively inhibits canalicular bile acid transport (Bolder et al., 1999). Ethinylestradiol-17β- glucuronide is secreted into bile by Mrp2 and then trans-inhibits Bsep from the luminal side of the canalicular membrane (Stieger et al., 2000). Cyclosporine, an MDR1 substrate, is a prototypical drug that can cause cholestatic liver injury by competitively inhibiting ATP dependent transporters such as BSEP and MRP2 (Bohme et al., 1993; Bohme et al., 1994; Kadmon et al., 1993) and it can inhibit intrahepatic vesicle transport, targeting, and function of BSEP to the canalicular membrane (Roman et al., 2003; Roman et al., 1990). Other drugs that can be associated with cholestasis, such as the endothelin antagonist bosentan, also inhibit the bile salt export pump, an effect that is enhanced by co-administration of the oral hypoglycemic agent glibenclamide (Fattinger et al., 2001). Troglitazone and troglitazone sulfate, the main troglitazone metabolites eliminated in bile, competitively cis-inhibit Bsep, which could lead to troglitazone-induced intrahepatic cholestasis and liver toxicity (Funk et al., 2001a; Funk et al., 2001b). Male rats are more susceptible to liver injury than female rats, probably due to higher formation rates of troglitazone sulfate (Kostrubsky et al., 2001). Although DILI is a clinically important and serious problem, the frequency of adverse drug reactions is low enough to suggest that these patients may also have genetic susceptibility factors. The *BSEP* variants, V444A and M677V, have been reported to consistently occur with frequencies of greater that 50% (Lang et al., 2006; Saito et al., 2002). The V444A variant has been found in patients with DILI (Lang et al., 2007) and intrahepatic cholestasis of pregnancy (Dixon et al., 2009; Meier et al., 2008) with greater frequency than controls. In contrast, preliminary study from Japan reported a lower frequency of the V444A variant in DILI (Kagawa et al.,

2012). Larger studies will be needed to clearly demonstrate an association of BSEP deficiency syndromes with a common *BSEP* variant.

6. Clinical Therapy

As described in the previous section and reviewed by Jacquemin (Jacquemin, 2012), BSEP mutations can fall into various classes, including nonsense mutations leading to truncated protein and missense mutations leading to endoplasmic reticulum-associated degradation (ERAD), mistrafficked protein, or protein with reduced functional activity. Ideally, clinical therapy should be guided by an individual's clinical presentation as assessed by immunohistochemistry, liver function tests, and genetic analysis (Jacquemin, 2012). Studies utilizing in vitro cell systems expressing different *BSEP/Bsep* mutations have provided a clearer understanding of how these genetic mutations affect the synthesized protein, thus allowing the development of targeted therapy. For example, the very low plasma membrane expression of the PFIC2 mutant, D482G, could be increased by treatment with low temperature, sodium butyrate (Figure 2) and sodium 4-phenylbutyrate (4-PB) in MDCK II, HEK293, or HepG2 cells (Hayashi and Sugiyama, 2007; Lam et al., 2007; Plass et al., 2004). These are believed to stabilize misfolded proteins which otherwise are subjected to ERAD and get trapped in the ER. Low temperature treatment had previously been shown to stabilize the CFTR ΔF508 mutant (Denning et al., 1992a). In vitro studies using rat *Bsep* mutants of the human mutations G238V, D482G, G982R, R1153C, and R1268Q all resulted in retention of Bsep in the ER to different extents (Wang et al., 2008). Ubiquitinylation with over expression of E3 ubiquitin ligases shortened the half life of both the wild type protein and the already short half life of the PFIC2 mutant, D482G, however small amounts of the mutant protein was still able to reach the plasma membrane (Wang et al., 2008). This and other studies also suggest that the residence time on the cell surface of the common D482G and E297G mutant proteins is greatly reduced due to accelerated internalization, reduced recycling or targeting of the endocytosed protein for degradation. These observations provide the rational for attempts to "rescue" the mutant proteins with small molecules (Hayashi and Sugiyama, 2009; Wang et al., 2008). However, it should be noted that additional studies would need to verify that an increase in plasma membrane localization of BSEP by drug chaperones would also translate to an increase in transporter function. Indeed, in vitro studies demonstrate that 4-phenylbutyrate (4-PBA) can increase the cell surface expression of PFIC2 mutant proteins D482G and E297G in MDCK cells and stimulate bile salt secretion and Bsep expression in vivo in rats (Hayashi and Sugiyama, 2007). This suggests that therapeutic intervention may aid in stabilizing BSEP at the plasma membrane. This was recently shown to be true in a child suffering from PFIC2 (Gonzales et al., 2012a). Phenylbutryate therapy was started as a final option for stabilizing the disease in this 10 year old child who had suffered since the age of one. After 5 months of therapy serum bile acid concentrations and pruritus had improved, as well as liver function tests. A liver biopsy performed after 3 months demonstrated canalicular localization of BSEP which had not been present before therapy was initiated (Figure 3). Discontinuation of the therapy resulted in reversion of the improvement. A preliminary report from the same group finds that 4-PBA decreases pruritus and serum bile acid concentrations, and improves liver function within 3

months of treatment in 3 PFIC2 children harboring a least one missense mutation (A257V, G982R and T1210P) (Gonzales et al., 2012b).

Additional therapeutic interventions could include drugs such as aminoglycosides and PTC124 to induce read through premature stop codons and treatment with nuclear receptor agonists (6-ethyl CDCA, fibrates statins) might be used to increase gene transcription of BSEP (Boyer, 2005; Gonzales and Jacquemin, 2010; Jacquemin, 2012; Trauner et al., 2005). The search for additional pharmacological chaperones that correct protein folding and trafficking defects in BSEP mutants continues.

One major hurdle that the pharmaceutical industry faces is the frequency with which drugs can cause drug-induced liver injury (DILI), where one of the major risk factors is inhibition of BSEP (Dawson et al., 2012; Greer et al., 2010). A recent study utilized a computational approach combined with an in vitro membrane vesicle assay to build a BSEP inhibition model (Warner et al., 2012). Although the algorithms are not perfect, their results show that lipophilicity and molecular size are significantly correlated with BSEP inhibition. In addition, Vertex Pharmaceuticals have investigated the ability of VX-809, a CFTR corrector, to partially correct the ΔF508 mutation commonly seen in cystic fibrosis (Van Goor et al., 2011). This drug improved chloride secretion and ER processing of CFTR in cultured human bronchial cells. Perhaps similar drugs can be developed to improve the misfolding of BSEP in common mutations leading to PFIC2.

Conclusions

The discovery of an ATP dependent bile salt transporter at the canalicular domain and its subsequent cloning (*ABCB11*) have provided the foundation for studies of BSEP's molecular regulation and cellular trafficking. While much has been learned of the effects of genetic mutations and polymorphisms, a more thorough understanding of the cellular determinants of the apical expression of this transporter both in health and in cholestatic liver disease remains to be elucidated. New therapeutic strategies for both genetic and acquired cholestatic liver diseases will depend on these future advances.

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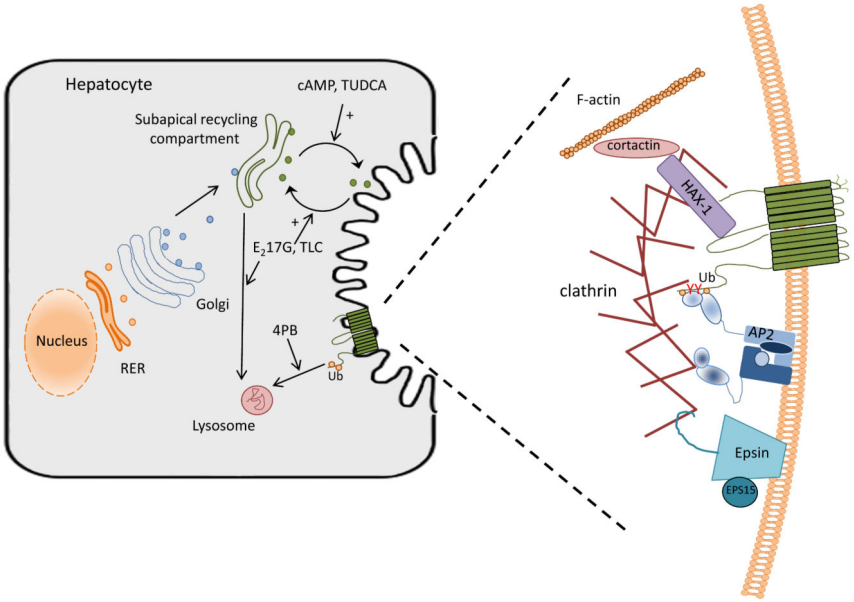

Fig 1.

Biosynthesis, trafficking and regulation of BSEP in the hepatocyte. (A) After post-translational modification in the Golgi, BSEP is trafficked to a subapical recycling compartment. Traffic to and from this endosomal compartment is highly regulated through various kinase pathways (Roma et al., 2008). Notably, cAMP and tauroursodeoxycholate treatment result in increased movement to the canalicular plasma membrane, whereas estradiol 17-glucuride and taurolithocholate treatment lead to increased endocytosis back to the recycling endosome and, perhaps eventually, to the lysosome for degradation. Phenylbutyrate (PB) may act by altering the rate of ubiquitination (Ub) and lysosomal degradation of BSEP. (B) Internalization of BSEP from the plasma membrane is a clathrin-dependent process involving many interacting partners. AP-2 has been shown to bind to a tyrosine containing motif in the C-terminus of Bsep which allows for endocytosis by a clathrin dependent mechanism (Hayashi et al., 2012; Lam et al., 2012). 4-PB has been shown to decrease the expression of the α-adaptin subunit of AP2, thus altering the ability of AP2 to interact with BSEP and decreasing endocytosis of the transporter (Hayashi et al., 2012). Ubiquination of the C-terminus of BSEP has also been shown to influence the cell surface expression of the transporter (Hayashi and Sugiyama, 2009). HAX-1 binds to the NBD domain of BSEP and with cortactin and has been shown to be involved in BSEP internalization from the plasma membrane (Ortiz et al., 2004). Epsin and EPS15 are important to the formation of the clathrin coated vesicle and, thus can influence the endocytosis of BSEP from the canalicular membrane (Ortiz et al., 2004). Regulation of these complexes through signaling pathways and by such compounds as phenylbutyrate may provide mechanisms for therapeutic intervention in various forms of cholestasis.

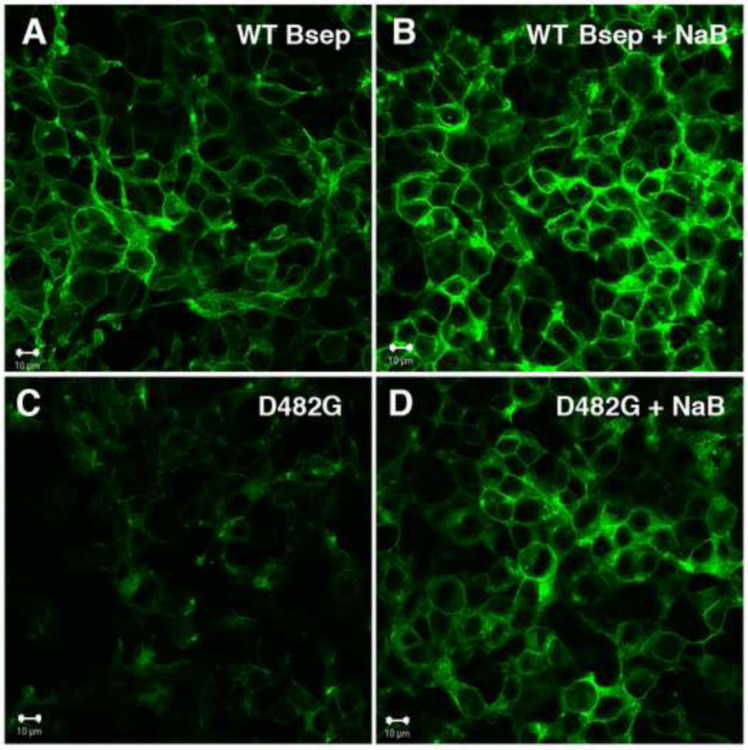

Figure 2.

Sodium butyrate increases the plasma membrane localization of Bsep-GFP. HEK293 cells were transfected with WT Bsep-GFP (A and B) or D482G-GFP (C and D) and confocal microscopy was used to monitor the plasma membrane expression of the constructs after treatment with sodium butyrate (NaB) for 24 hr. (A) DMSO control treated cells express WT Bsep-GFP primarily on the plasma membrane. (B) Similar localization was seen after NaB treatment. (C) Cells transfected with mutant D482G Bsep-GFP show little GFP fluorescence due to degradation of the protein (see details in (Lam et al., 2007). (D) Treatment with NaB resulted in stabilization of the protein and cells show increased plasma membrane expression of the D482G mutant of Bsep-GFP.

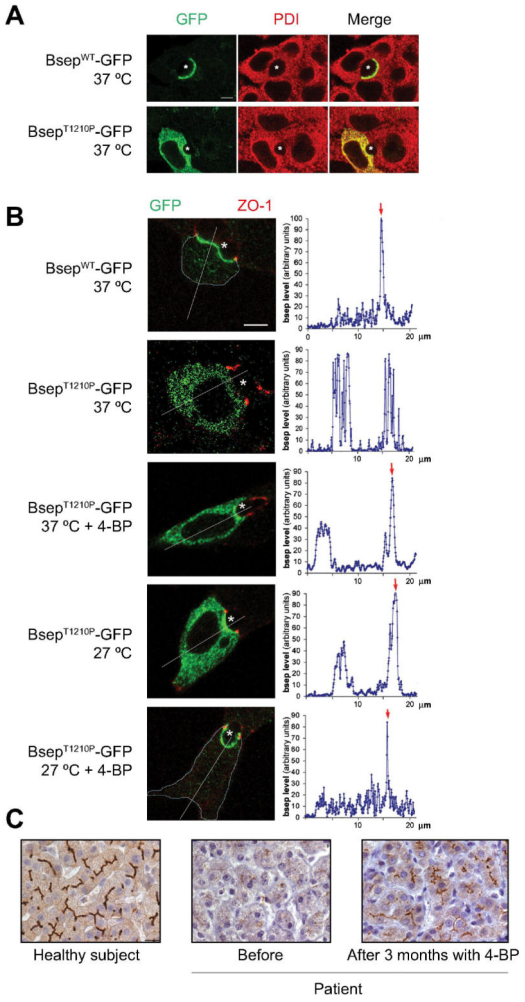

Figure 3.

In vitro studies of mutant Bsep^{T1210P}–GFP in a polarize cell line and the effect of 4-PB in a PFIC2 child expressing this mutation. (A) Immunofluorescent localization of GFP constructs of WT and mutant BSEP (green) and protein disulfide isomerase (PDI) (red) in Can 10 cells. (B) 4-PB and low temperature increase canalicular membrane expression of Bsep^{T1210P}–GFP (green). The apical membrane was delineated with immunofluorescent labeling of the tight junction protein, zonula occludens 1 (ZO-1) (red). Quantification of Bsep–GFP levels is shown in graphs to the right. Red arrows indicate canalicular fluorescence reinforcement, when present. A thin blue-grey line was drawn on confocal images to delineate cell limits, when they were hardly visible. * canaliculus; bar: 5 µm. (C) BSEP immunostaining of human liver sections: healthy subject (left), patient before (middle) and after 3 months with 4-PB therapy (right). Bar: 30 µm. (Reproduced with permission from (Gonzales et al., 2012a)).