Levels of plasma membrane expression in progressive and benign mutations of the bile salt export pump (Bsep/Abcb11) correlate with severity of cholestatic diseases

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THE BILE SALT EXPORT PUMP (BSEP) is a liver-specific glycoprotein that functions at the apical (canalicular) surface as an ATP-dependent bile salt export pump (1, 5, 20). BSEP belongs to the ATP-binding cassette (ABC) transporter protein family. Rat Bsep is a 1,321-amino acid protein with 12 putative membrane-spanning domains and 2 nucleotide-binding domains. When first synthesized in the endoplasmic reticulum (ER) it is expressed as a core-glycosylated (immature) protein, and intracellular protein that is expressed at the cell surface and that strategies to stabilize cell surface mutant protein may be therapeutic.

ATP-binding cassette; progressive familial intrahepatic cholestasis type 2; benign recurrent intrahepatic cholestasis type 2; intrahepatic cholestasis of pregnancy; taurocholate transport

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Fig. 1. Missense mutations identified in Bsep. A: putative topological model of Bsep and the missense mutations associated with progressive familial intrahepatic cholestasis type 2 (PFIC2), benign recurrent intrahepatic cholestasis type 2 (BRIC2), and intrahepatic cholestasis of pregnancy (ICP). The functional domains (Walker A, B, and C) are indicated. PFIC2 (D482G, E297G), BRIC2 (A570T, R1050C), and ICP (N591S) mutations that are investigated in this study are indicated in italics. The mutation is designated as the original amino acid residue (1st letter), its location (number), and the amino acid to which it was changed (2nd letter). PFIC2 (⊗); BRIC2 (●) and ICP (●) missense mutations have been identified. V444A (●) has been identified as a polymorphic site (14). B: alignment of mutations in this study indicates conservation between different species.

Contrast to these conclusions, we find that all five green fluorescent protein (GFP)-tagged mutant proteins, including D482G and E297G, traffic to the cell surface as detected by confocal microscopy and by biotinylation of plasma membrane Bsep when expressed in MDCK and HEK293 cells, respectively. Our findings support the conclusion that the expression levels of mutant proteins in the cell and at the cell surface are significantly lower than those of wild-type (WT) protein but suggest further that these mutant proteins, while mature (e.g., fully glycosylated), are less stable at the plasma membrane. Since ATPase and bile salt transport activity when expressed in Sf9 cells are also normal (with the exception of the E297G mutant) the severity of the clinical phenotype correlates most closely with mutation-induced defects in cell surface expression of the mature protein. Therefore, our findings imply that therapeutic strategies directed toward enhancing cell surface expression of the BSEP mutants might be therapeutic for some of these hereditary cholestatic diseases.

MATERIALS AND METHODS

DNA constructs. To examine the subcellular localization of the Bsep protein, full-length Bsep cDNA from rat liver (kindly provided by Peter Meier, University Hospital, Zurich, Switzerland) was cut out of the pBK-CMV vector and subcloned into pEGFPN1 (Clontech Laboratories, Palo Alto, CA), using 5'-HindIII and 3'-AgaI sites. All point mutations (D482G, E297G, A570T, R1050C, N591S) were introduced with the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). All immunofluorescence and quantitative Western blot analyses were performed with these constructs transfected into MDCK or HEK293 cells. Rat ubiquitin tagged with hemagglutinin (HA) in pcDNA vector was kindly provided by Dr. Pietro De Camilli (Yale University).

Cell cultures and transfections. MDCK and HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, high glucose) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 1% penicillin-1% streptomycin (Invitrogen) at 37°C. For transient transfection, cells were plated at 70% confluence in a 12-well plate for confocal microscopy and in a 24-well plate for biochemical studies 16–24 h before transfection. Transfection was carried out with LipofectAMINE 2000 according to the manufacturer’s instructions (Invitrogen). Experiments were performed 48 h after transfection.

Cell surface expression. Transiently transfected HEK293 cells expressing Bsep or mutants were grown for 48 h after transfection in a six-well plate and washed three times with cold PBS containing 0.1 mM CaCl₂ and 1 mM MgCl₂ (PBSMc). The cell surface was biotinylated with 1 mg/ml membrane-impermeant sulfo-NHS-S-S-biotin (Pierce Chemical, Rockford, IL) for 1 h at 4°C. Unreacted biotin was quenched with cold PBS containing 0.1% BSA. Cell lysates were directly lysed for 15 min with RIPA buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Cleared lysates containing equal amounts of proteins were incubated with immunoperoxidase immobilized streptavidin at 4°C overnight (Pierce Chemical). Streptavidin beads were washed four times with PBSMc containing 0.5% wt/vol) sodium deoxycholate. The bound proteins were eluted with 1× Laemmli buffer and separated by SDS-PAGE. Quantification of cell
surface expression was performed by Western immunoblotting using 1:5,000 anti-Bsep polyclonal (from Dr. Victor Ling, BC Cancer Research Center, Vancouver, British Columbia, Canada) or 1:1,000 anti-GFP monoclonal (Clontech) antibody. An aliquot of the original lysate was also subjected to immunoblotting using the anti-Bsep antibody in order to determine the total expression of Bsep in the cells. To control for possible cell density differences the original lysate and the biotinylated protein fraction were immunoblotted with Multi-Analyt software (Bio-Rad, Hercules, CA). The WT control signal determined by densitometric analysis of the immunoblots with Multi-Analyst software (Bio-Rad, Hercules, CA). The WT control signal was taken as 100.0 ± SD, and the Bsep mutation signals were normalized to it.

In vitro peptide N-glycosidase F and endoglycosidase H$_{1}$ treatment. Cells expressing WT and mutant Bsep were digested with peptide N-glycosidase (PNGase F) and endoglycosidase H$_{1}$ (endoH) according to the manufacturer’s instructions (New England Biolabs, Ipswich, MA).

Immunofluorescence and confocal microscopy. Cells expressing Bsep-GFP were washed two times in PBS/0.1% BSA, fixed for 20 min with 4% paraformaldehyde, and rewash in PBS/0.1% BSA. The fixed cells were then permeabilized with 0.05% Triton X-100 for 20 min. The cells were washed twice at room temperature in PBS containing 1% BSA and then incubated for 1 h in the same medium containing the appropriate primary antibody: Rab11 (recycling endosomes; Zymed Laboratories), HA (Clontech), and ZO-1 (Zymed). After the cells were washed, primary antibodies were detected by reaction with 1:500 Alexa conjugated secondary antibodies. Nuclei were stained with Topro3 (1:5,000 for 5 min). Samples were visualized with a Zeiss LSM-510 Meta laser-scanning microscope (Carl Zeiss, Thornwood, NJ).

Immunoprecipitation and detection of ubiquitinated Bsep. Stably transfected D482G HEK293 cells were treated with and without 2 μM MG-132 for 16 h. Cells were lysed under denaturing conditions with 2% SDS in 100 μl of lysis buffer [10 mM Tris·HCl pH 7.4, 100 mM NaCl, 1% Triton X-100, and protease inhibitor cocktail (Roche)] for 10 min and diluted further with 900 μl of lysis buffer. The lysate was cleared by centrifugation at 16,000 g for 10 min at 4°C. The lysate was precleared with 1 μg of mouse IgG antibody and 30 μl of protein A/G Plus beads (Pierce). The precleared cell lysate was immunoprecipitated with 1 μg of GFP antibody in 30 μl of protein A/G Plus beads and eluted with loading buffer after three washes with lysis buffer. The blot was probed with anti-Bsep (from Dr. Victor Ling) and anti-ubiquitin (Santa Cruz) antibodies.

Production of recombinant baculovirus. To characterize the functional activity of the Bsep protein, recombinant baculovirus carrying the rat Bsep gene was generated. For this purpose, full-length Bsep cDNA was cloned into pFastBac1, and Sf9 cells were infected and cultured according to the procedure described by Invitrogen.

ATPase activity. Sf9 cells infected with recombinant virus (mock, WT, D482G, E297G, A570T, R1050C, and N591S) were harvested, and cell membranes were prepared as described previously (23). ATPase activity was measured as described previously by determining the liberation of inorganic phosphate (P$_{i}$) from ATP with a colorimetric assay (23). The specific ATPase activity of the mutants was compared with WT in the presence or absence of 200 μM vanadate. The specific ATPase activity was normalized to the amount of protein as determined by Western immunoblotting. Data represent means ± SD of triplicate determinations. Statistical significance was tested with a paired t-test, assuming significance with a P value of <0.05.

Taurocholate uptake. Uptake of [3H]taurocholate into the Sf9 cell membranes was measured as previously described (2). Data represent means ± SD. Statistical significance was tested with a paired t-test, assuming significance with a P value of <0.05.

RESULTS

Effect of BSEP mutations on subcellular localization of Bsep-GFP. Many missense mutations that cluster around the Walker A and B motifs have been identified in the nucleotide-binding domain of BSEP (Fig. 1A). The mutations we chose to study in the rat gene are in regions highly conserved among different species (Fig. 1B). The subcellular distributions of D482G, E297G, A570T, R1050C, and N591S were examined first because intracellular accumulation of misfolded proteins has been shown for many diseases. WT and mutant Bsep-bearing COOH-terminal GFP epitopes were expressed transiently in MDCK cells and HEK293 cells and visualized with confocal microscopy. In contrast to the intracellular ER-like distribution pattern observed for delta F508 cystic fibrosis transmembrane conductance regulator (CFTR), both WT and mutant Bsep were detectable at the cell surface and inside the cell in MDCK (Fig. 2A) and HEK293 (Fig. 2B) cells. The exchange of Bsep between the canicular membrane and Rab11a-positive endosomes has been documented (29). We also found that the WT and mutant Bseps distribute to Rab11a-containing endosomes (Fig. 3), suggesting that trafficking of Bsep mutants has reached the endocytic pathway. These results suggest that trafficking to the plasma membrane is not affected by these mutations and is similar in both polarized and nonpolarized cell types.

Reduced plasma membrane expression of mutant Bsep proteins. It is further postulated that degradation is likely to be responsible for the reduced expression of mutant Bseps. Quantitative RNA analysis by real-time PCR indicated that Bsep transcript levels were not significantly different in cells expressing WT or mutant Bsep (Fig. 4A). To compare the level of protein expression of Bsep in the plasma membrane, cell surface proteins were biotinylated and subjected to streptavidin bead pull-down and Western immunoblotting using GFP antibody to detect Bsep-GFP protein. We detected only one band for the biotinylated protein, and the mobility of the biotinylated species corresponds to the mature fully glycosylated form of BSEP (band C) (Fig. 4B). The level of cell surface expression of Bsep protein was quantitated by densitometry and determined to be in the following order: WT (100%) > N591S (75.6 ± 15.6%) > E297G (38.5 ± 12.6%) ~ R1050C (35.6 ± 14.5%) ~ A570T (29.5 ± 8.8%) >> D482G (5.7 ± 2.3%). Western blots of total protein lysates showed two major bands with the top band (band C) representing mature protein and the bottom band (band B) representing immature protein (see Fig. 5A, glycosylation status, for further explanation). The total expression (cell surface and intracellular proteins) also showed changes similar to those in cell surface expression: WT (100%) > N591S (73.6 ± 4.3%) > E297G (33.7 ± 20.4%) ~ R1050C (26.7 ± 16.0%) ~ A570T (11.9 ± 5.2%) >> D482G (2.5 ± 2.0%) (Fig. 4B). These findings suggest that the mutations reduced the expression level but did not significantly impair the processing of the Bsep protein.

Glycosylation status indicates that mutant proteins are mature. As shown in Fig. 5A, Bsep is modified by the addition of an N-linked carbohydrate. The high-molecular-mass band C is resistant to endoH but sensitive to treatment by PNGase F. This indicates that band C represents the mature form of Bsep. The second lower-molecular-mass band (band B) is sensitive to endoH and PNGase F digestion, indicating that this band
D482G protein (band C) compared with WT protein, suggesting that mutant band C. Mutant proteins displayed less mature protein band C form (protein is largely expressed as the mature, fully glycosylated Bsep transiently transfected with pEGFPN1 (control), rat Fig. 2. Localization of Bsep proteins in MDCK and HEK293 cells. Cells were transiently transfected with pEGFPN1 (control), rat Bsep-GFP [wild type (WT)], D482G-GFP, E297G-GFP, A570T-GFP, R1050C-GFP, and N591S-GFP constructs. A: green fluorescent protein (GFP)-tagged WT Bsep is present in the apical membrane of Madin-Darby canine kidney (MDCK) cells. In contrast, 4 of the mutant GFP-tagged Bsep proteins are variably localized to both intracellular and apical membranes, whereas localization of N591S-GFP is similar to WT. Tight junctions are labeled for ZO-1 in red. The z-section image is also shown. AP, apical membrane; BL, basolateral membrane. B: in HEK293 cells, WT Bsep localizes to the plasma membrane, while mutant proteins are localized to both plasma membrane and intracellular compartments. Bars = 5 μM.

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represents the core-glycosylated form of the protein. WT protein is largely expressed as the mature, fully glycosylated form (band C). Mutant proteins displayed less mature protein (band C) compared with WT protein, suggesting that mutant proteins are not stable at the cell surface and may be degraded.

To confirm that the decrease of Bsep expression is mediated by ubiquitin-proteasome degradation, the effect of the proteasome inhibitor MG-132 on the steady-state levels of mature (complex glycosylated, band C) and immature (core glycosylated, band B) Bsep was assessed by immunoblot analysis of HEK293 cells expressing WT and mutant protein. Incubation with MG-132 increased the high-molecular-mass smear punctuated by discrete bands evident near the top of the gel (Fig. 5B). These species presumably represent ubiquitinated forms of Bsep. We colocalized the D482G mutant with endogenous ubiquitin and with transfected rat ubiquitin tagged with hemagglutinin (Fig. 5C). After incubation with MG-132 for at least 16 h, aggregates of Bsep resembling aggresome structures formed in the perinuclear region. These aggregates colocalized with HA antibody, indicating that Bsep degradation can be inhibited by proteasome inhibitors. To further confirm that Bsep is ubiquitinated, we immunoprecipitated D482G molecules from stably transfected cells and blotted the immunoprecipitates with an anti-ubiquitin antibody (Fig. 5D). A smear of protein ladder was detected consistent with oligo-ubiquitination. MG-132 treatment increased both the D482G protein and its ubiquitinated form. These results indicate that the proteasome is involved in the turnover of D482G protein.

Reduced temperature and chemical chaperones induce accumulation of mutant Bsep. To investigate whether chemical chaperones could stabilize the D482G mutant, we found that reduced temperature, sodium butyrate, and sodium 4-phenylbutyrate could enhance the expression of the mature D482G protein (band C) and surface expression in stably transfected HEK293 cells (Fig. 6). Recently, Hayashi and Sugiyama (7) also showed that sodium 4-phenylbutyrate enhances the expression of cell surface human D482G protein in MDCK cells. These results confirm that 4-phenylbutyrate has similar effect in stabilizing D482G mutant with human or rat gene.

Functional significance of Bsep mutations. To study Bsep function, we overexpressed WT and mutant Bsep in Sf9 insect cells and examined the ATPase activity and taurocholate transport in isolated membrane vesicles. The expression of the Bsep protein was detected by immunoblotting with anti-Bsep protein polyclonal antibody (Fig. 7A). Bsep immunoreactive protein was expressed in the membrane fraction of the Bsep baculovirus-infected Sf9 cells and was not present in the baculovirus negative control (pFastBac1-Gus). Membrane vesicles containing WT Bsep (17.1 ± 2.5 nmol P_1·min^−1·mg protein^−1) and mutant N591S Bsep (18.4 ± 1.53 nmol P_1·min^−1·mg protein^−1) showed slightly higher basal ATPase activity than membrane vesicles containing the mock-transfected control (12.3 ± 0.67 nmol P_1·min^−1·mg protein^−1) (Fig. 7B). The ATPase activities of membrane vesicles expressing WT and Bsep variants were stimulated by addition of the bile acid substrate taurocholate and inhibited by addition of a specific inhibitor, orthovanadate (Fig. 7B). We observed no stimulation by taurocholate or inhibition by orthovanadate in membrane vesicles containing mock-transfected pFastBac1-Gus, indicating that WT and mutant Bsep are capable of ATP hydrolysis and stimulation by its substrate.

Bsep-expressing Sf9 cell vesicles showed marked ATP-dependent stimulation of [3H]taurocholate transport (~50 pmol·mg^−1·10 min^−1) (Fig. 7C). The membrane vesicles expressing D482G, A570T, R1050C, or N591S showed similar levels of taurocholate uptake compared with WT Bsep. The E297G mutation reduced the taurocholate uptake of Bsep to a level similar to membrane vesicles from mock-treated cells. Although the mutations have significant effects on Bsep protein expression, the catalytic functions of the protein are maintained in all mutants except the E297G-expressing membrane vesicles.
DISCUSSION

When five BSEP mutants representing different forms of clinical cholestatic phenotypes were introduced into rat Bsep and expressed in MDCK or HEK293 cells, we found that they traffic properly to the plasma membrane. In addition, their plasma membrane expression levels correlate closely with the respective clinical phenotypes from which they were derived. A PFIC2 mutation (D482G) is highly unstable (~5% of WT), while the BRIC2 variants A570T and R1050C are expressed at the plasma membrane at intermediate levels (~25% of WT). In contrast, the surface expression of the ICP variant N591S is close to the level of the WT protein (~70%). We have demonstrated in this study that the stability of mutant proteins may be regulated by the ubiquitin-proteasome pathway. Ubiquitination, a reaction whereby ubiquitin molecule(s) are covalently attached to substrate proteins, plays a crucial role in

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Fig. 3. Localization of WT and mutants variably distributed to plasma membrane and Rab11a-recycling endosomes in HEK293 cells. Cells were transfected with rat Bsep-GFP (WT), D482G-GFP, E297G-GFP, A570T-GFP, R1050C-GFP, and N591S-GFP constructs and were examined for GFP by confocal laser microscopy. WT-GFP (green) was mainly detected in the apical membrane and colocalized with markers for recycling endosomes (Rab11a; red). D482G, E297G, A570T, and R1050C mutants partially colocalized with recycling endosome marker Rab11. N591S was mainly expressed on the membrane surface, a pattern similar to the WT protein. Bars = 5 μm.

Fig. 4. mRNA expression levels are similar between WT and Bsep mutants but protein expression levels are reduced in mutants of HEK293 cells. A: quantitative polymerase chain reaction of mRNA expression levels of WT and mutant Bsep. Bsep gene expression was normalized to GAPDH. D4, D482G; E2, E297G; A5, A570T; R10, R1050C; N5, N591S. N1 is GFP green fluorescence control. Data represent means ± SD of 4 determinations. B: Bsep mutations do not impair processing of the mature protein. Left: immunoblots of HEK293 cells expressing WT and mutants in cell lysates or after membrane-selective cell surface biotinylation. Mature, fully glycosylated Bsep (band C) and immature, core-glycosylated (band B) protein are indicated. Right: densitometric quantitation of expression of surface and total protein represents means ± SD of 3 experiments. *P < 0.05, D482G, E297G, A570T, R1050C, and N591S compared with WT control.
the degradation and turnover of certain membrane proteins. Ubiquitination was found to be required in the degradation of CFTR (25, 31), P-glycoprotein (32), V2 vasopressin receptor (16), platelet-derived growth factor receptor (18), and epithelial Na\(^+\) channel (26) before lysosomal or proteasomal degradation. This study shows that the D482G mutant is ubiquitinated and that proteasome inhibition increases the level of the mutant protein, thereby providing a means to regulate Bsep stability.

Our findings in this study are also consistent with in vivo expression of the Bsep protein in patients with PFIC2, BRIC2, and ICP variants as analyzed by immunohistochemistry. Since it is not possible to accurately measure the level of protein expression by immunostaining in these cases, the present study provides further in vitro evidence that the distinction between recurrent and progressive forms of intrahepatic cholestasis is largely based on the level of surface expression of Bsep protein. This conclusion assumes that the processing of Bsep in HEK293 cells overexpressing the protein reflects events occurring in hepatocytes in these cholestatic patients. We believe this is a reasonable assumption because liver biopsies from patients with PFIC2 mutation(s) have detected an absence of Bsep, while liver biopsies from a BRIC2 patient displayed normal canalicular expression (19). A third patient with severe ICP was found to have combined homozygous alterations of a BSEP polymorphism (V444A) and a MDR3 missense mutation, possibly explaining the early onset and severity of the ICP (10).

Our results confirm previous results from this lab (30) and others (8, 19, 22) that have described a low expression of the PFIC2 mutant D482G in human, rat, and mouse Bsep following transfection into different mammalian cell lines. With the exception of the E297G variant, all of the studied variants

![Figure 5](image_url)

**Fig. 5.** Bsep mutants are processed to mature protein, and misprocessed mutant Bsep proteins are degraded by proteasomes. **A:** in vitro deglycosylation of Bsep protein with peptide N-glycosidase F (F) or endoglycosidase H (H) indicates that band C is fully glycosylated whereas the lower-molecular-mass band is core glycosylated; asterisk (*) presumably represents uncut core-glycosylated protein. Twenty-five micrograms of protein was used for each reaction, except that 100 μg of D482G protein was used for digestion to detect expression (**). **B:** degradation of WT and Bsep mutants is inhibited by proteasome inhibitor MG-132. HEK293 cells were transfected as described and treated with (+) and without (−) MG-132 (2 μM) for 16 h before lysis and separation. MG-132 increased the expression of immature, core-glycosylated protein (band B) of WT and mutants to different extents. **C:** Bsep-D482G-GFP (a) colocalizes with transfected hemagglutinin (HA)-tagged ubiquitin (b) with HA antibody. c: Merged image showing strong perinuclear staining and colocalization. Bar = 5 μm. **D:** Western blots of total lysates or immunoprecipitates (IP) of D482G stably transfected HEK293 cells treated with and without MG-132 using anti-ubiquitin (ub) antibody or Bsep antibody (Bsep).

![Figure 6](image_url)

**Fig. 6.** Temperature and chemical chaperones rescue mature D482G mutant protein. **A:** stably transfected WT (top) and D482G (bottom) in HEK293 cells were incubated at 37°C or shifted to 27°C overnight. There was a marked increase in plasma membrane expression of the D482G-GFP mutant compared with slight increase in plasma membrane expression of WT-GFP. Western immunoblotting shows the increase in expression of mature and immature protein of D482G (bottom) compared with little change in expression of WT (top) after temperature rescue. **B:** chemical chaperones sodium butyrate (NaB) and sodium 4-phenylbutyrate (NaPB) and DMSO control (Ctl) were used to treat WT (top) and D482G (bottom) stably transfected HEK293 cells for 24 h. NaB and NaPB enhanced the expression of D482G at the plasma membrane and increased the expression levels of mature and immature protein (bottom).
retained normal bile acid transport activity in Sf9 insect cell membrane vesicles. In contrast, in membrane vesicles from HEK293 cells that overexpress the human E297G variant, taurocholate uptake activity is retained with the same transport efficiency as WT Bsep (8). In addition, in patients the E297G mutation has been associated with different clinical phenotypes as well as in healthy relatives (9, 19, 28). On the basis of this study and others, it appears that the biochemical data in the human HEK293 cells indicate that E297G mutation results in some protein expression and has residual bile acid transport activity. However, the absence of bile acid transport activity in Sf9 membrane vesicles expressing the E297G variant suggests that the mechanism for the E297G-associated defect is likely to be more complex. Differences in experimental design and species-specific effects on the function of this variant transporter cannot be ruled out.

Although there have been reports that some BRIC2 patients may progress to a PFIC2 phenotype, BRIC2 is usually a milder intermittent cholestatic disease that affects adult patients (13). This has led to the speculation that a “second hit” that impairs trafficking-expression or function is necessary to bring out the cholestatic phenotype or perhaps lead to a more severe progressive phenotype. The nature of the second hit is not clear, but earlier reports imply that recurrent infections may play a role, perhaps by eliciting cytokine responses that downregulate BSEP and possibly other canalicular transport proteins as has been shown experimentally (4). Administration of lipopolysaccharide or 17α-ethinylestradiol to mice showed a decrease in Bsep mRNA and protein levels, indicating that additional factors affecting transcriptional and posttranscriptional activities may contribute to the cholestatic phenotype in some of these patients (3, 6).

The finding that most mutants retained normal bile acid transport is therapeutically important, if confirmed in the human mutations, since strategies directed toward maintaining their cell surface stability would be expected to improve the degree of cholestasis. Reduced temperature and 4-phenylbutyrate have been demonstrated in this study and by others (7) to increase mature and cell surface protein expression for the D482G mutant.

In summary, BSEP is the major determinant of bile salt secretion, and mutations in this gene have been linked to different cholestatic phenotypes. We have determined that missense substitutions identified in patients differentially affect the expression and function of the Bsep protein when introduced into rat Bsep and expressed in mammalian cells. These in vitro effects correlate with the severity of their respective clinical phenotypes. Our data also indicate that level of cell surface expression correlates inversely with the severity of the disease-associated mutations. These findings should facilitate the clinical interpretation of these missense variants and direct future clinical strategies.

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