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ATPase Class I Type 8B Member 1 and Protein Kinase C ζ Induce the Expression of the Canalicular Bile Salt Export Pump in Human Hepatocytes

FRANK CHEN, EWA ELLIS, STEPHEN C. STROM, AND BENJAMIN L. SHNEIDER

Children's Hospital of Pittsburgh of UPMC and the Departments of Pediatrics [F.C., B.L.S.], and Pathology [E.E., S.C.S.], University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15224

ABSTRACT: The exact molecular mechanism(s) of the disease that results from defects in the ATPase Class I Type 8B Member 1 gene remains controversial. Prior investigations of human ileum and in intestinal and ovarian cell lines have suggested that familial intrahepatic cholestasis 1 (FIC1) activates the farnesoid X-receptor (FXR) via a pathway involving protein kinase C ζ (PKCζ). Translational investigations of human liver from individuals with FIC1 disease have been confounded by secondary affects of progressive cholestatic liver disease and limited numbers of samples for analysis. These studies, performed in primarily derived human hepatocytes, circumvent this issue. The canalicular bile salt export pump (BSEP) served as a downstream target of FXR. The siRNA-mediated silencing of FIC1 in human hepatocytes led to a reduction in both human BSEP promoter activity and BSEP protein expression, which correlated with a reduction in FXR expression and redistribution of its localization from the nucleus to the cytoplasm. These changes in BSEP expression could be reproduced by altering the expression of PKCζ, with a positive correlation of PKCζ activity and BSEP expression. Overall, these findings support the hypothesis that FIC1 enhances FXR signaling via a PKCζ-dependent signaling pathway. **(*Pediatr Res* 67: 183–187, 2010)**

Progressive familial intrahepatic cholestasis type 1 (PFIC1, also known as FIC1 disease or Byler's disease) is the result of mutations in the ATPase Class I Type 8B Member 1 gene, which is also referred to as FIC1 (1). FIC1 is expressed in a wide variety of tissues, and as such, FIC1 disease has potential systemic manifestations (2). Cholestasis is the primary disease manifestation in FIC1 disease and is presumed to be the result of diminished canalicular excretion of bile salts. The precise pathophysiology of FIC1 disease remains controversial, with two distinct proposed mechanisms. FIC1 expression enhances aminophospholipid transport across plasma membranes, presumably by facilitating flipping of these molecules from the outer to inner hemileaflet of the lipid bilayer (1,3,4). It has been suggested that alterations in aminophospholipid distribution in the lipid bilayer alter resident protein function and intramembrane residency [e.g. the canalicular bile salt export pump, BSEP (ABCB11)] and thus generates the reported pathophysiology (5). An alternative proposed pathophysiology is that FIC1 signals through protein kinase C (PKC)-ζ to phosphorylate and activate the bile acid-binding nuclear receptor, farnesoid X receptor (FXR; 6). Expression of BSEP and the ileal apical sodium-dependent bile acid transporter (ASBT) are modulated by FXR (7–9). It is proposed that, in FIC1 disease, BSEP expression is reduced and ASBT is induced, which would lead to the clinically observed cholestasis (10).

Testing these hypotheses regarding the pathophysiology of FIC1 disease in cell culture, mouse models, and humans with FIC1 disease has yielded conflicting findings. Molecular events in FIC1 disease have been examined in tissues from children affected by this disease. Altered FXR signaling was described in the ileum of three children with FIC1 disease compared with two children with other forms of intrahepatic cholestasis (10). ASBT protein abundance was increased in FIC1 disease relative to the other forms of cholestasis. Investigation of hepatic BSEP expression in children with FIC1 disease has yielded inconsistent results (11,12). There are a variety of complicating factors in the analysis of BSEP expression in human liver disease, including secondary effects of cholestasis and/or advanced liver disease and interindividual variability of BSEP expression (13).

In light of the difficulties in analysis of molecular events using human tissues, efforts have been undertaken to model FIC1 disease and study FIC1 function in cell culture. FIC1 silencing in the human colon carcinoma cell line, Caco-2, leads to diminished phosphorylation, nuclear localization, and activation of FXR with predictable effects on downstream targets like ASBT (10). FIC1 gain-of-function in the FIC1 deficient cell line, UPS, yields PKCζ-dependent activation of FXR (6). The effects of silencing FIC1 in hepatocyte cell lines have yielded conflicting results. In the human hepatoblastoma cell line, HepG2, FIC1 silencing leads to diminished BSEP promoter activity and reduced FXR signaling (10,14,15). Similar studies in primarily derived human hepatocytes reveal diminished BSEP function, although signaling via FXR does

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Correspondence: Benjamin Shneider, M.D., Division of Pediatric Gastroenterology, Hepatology and Nutrition, Children's Hospital of Pittsburgh of UPMC, One Children's Hospital Drive, 4401 Penn Avenue, Pittsburgh, PA 15224; e-mail: benjamin.shneider@chp.edu

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Abbreviations: ASBT, apical sodium-dependent bile acid transporter; **BSEP,** bile salt export pump; **CDCA,** chenodeoxycholic acid; **CTFCS,** charcoal treated fetal calf serum; **FIC1,** familial intrahepatic cholestasis 1; **FXR,** farnesoid X receptor; **PKC,** protein kinase C; **SHP,** small heterodimer partner

not seem to be involved (16). The following studies have been performed in primarily derived human hepatocytes to further examine the hypothesis that FIC1 signals through PKCζ to activate BSEP via FXR.

METHODS

Cell culture. Normal human hepatocytes were obtained through the Liver Tissue Cell Distribution System, Pittsburgh, PA. The Institutional Review Board of the University of Pittsburgh approved this study. Liver cells were isolated by a three-step collagenase perfusion, as described previously (17). Liver cells, composed of 90% hepatocytes and 10% nonparenchymal cells by morphologic evaluation, had viability at plating of greater than 80%. Hepatocytes were suspended in hepatocyte maintenance medium (HMM; Lonza, Walkersville, MD), supplemented with 10 nM insulin, 10 nM dexamethasone, 100 U/mL penicillin G, 50 μg/mL gentamicin, 50 ng/mL amphotericin, and 5% FCS. HepG2 (HB-8065) cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in Eagle's minimum essential medium (Invitrogen) containing 2 mM l-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, and 10% FCS (Invitrogen). When FXR-ligand responses were being assessed, cells were transferred to a medium containing 0.5% charcoal-treated FCS (CTFCS) to minimize the effects of the bile acids found in FCS (6). GW4064 as an FXR ligand was generously provided by Dr. Song Li (University of Pittsburgh School of Pharmacy, Pittsburgh PA). Cells were plated on 6-well culture plates and cultured in 5% CO2 at 37°C for 24 to 48 h before transfection.

Plasmid constructs and antibodies. To study the effect of PKCζ on FIC1/FXR-mediated gene expression, transfection analyses were performed using the silencing expression plasmid construct pRNA-sihPKCζ (GenScript Corporation, Piscataway, NJ), which contained a small interfering PKCζ mRNA sequence, 5′-augaccaaauuuacgccauga-3′ (nucleotides 797–817), contained in the coding region of human PKCζ gene (GenBank accession number Z15108). In some experiments, human hepatocytes were treated for 2 h before harvest with a specific PKCζ inhibitor, PKCζ pseudosubstrate (PKCζ pseudo, myristoylated, 100 μM; EMD Biosciences, Inc., San Diego, CA). The wild-type human PKCζ (wtPKC) and human PKCζ dominant negative mutant (PKCζμ) plasmid constructs were obtained as generous gifts from Dr. Marc Bissonnette, University of Chicago, Chicago, IL (18). The FIC1 antisense plasmid construct pRNA-siFIC1 (siFIC1) and an antisense control plasmid construct pRNA-siScr (siScr) were purchased from GenScript Corporation (Piscataway, NJ; 10). A human FXR (hFXR) expression construct, pCMX-hFXR was a generous gift from Dr. David Mangelsdorf, University of Texas, Southwestern, Dallas, TX (19,20). The activity of a 0.2-kb BSEP promoter (−145 to +86) linked to a luciferase expression vector pSV0AL[Delta]5′ (p-145/Luc, generous gift from M. Ananthanarayanan, Mount Sinai School of Medicine, NY, NY) was used as a read out of the FXR functional status in the transfection assays (7).

The following sources of antibodies were used in this study: developed as previously described—FIC1 (6); from Santa Cruz Biotechnology, Inc, (Santa Cruz, CA)—antigen (catalogue number), BSEP (sc-74500); Na/K-ATPase (sc-48345); FXR (sc-25309); short heterodimer partner (SHP; sc-30169); Histone H1 (sc-8030); and PKCζ (sc-216); from Sigma Chemical Co., (St. Louis, MO)—Actin (A5441), PhosphoSer (P3430), PhosphoTyr (P3300), and PhosphoThr (P3555).

Transient transfection and dual luciferase assay. Cells (5 × 106/plate) were transiently transfected with 3 μg of plasmid constructs and 0.1 μg of pRL-TK containing a thymidine kinase promoter-driven Renilla luciferase gene (Promega, Madison, WI). Cells were suspended in 700 μL of PBS-containing DNA plasmid constructs, and transfection was accomplished by electroporation (11) at 0.22 kV and 0.95 μF × 1000 (Bio-Rad). After electroporation, the cells were resuspended in culture medium and returned to a plate for culturing for 48 h before dual luciferase assays (Promega). Luciferase activities were determined by the Dual Luciferase Reporter Assay System (Promega) as described by the manufacturer's protocol, using a 20/20n Luminometer (Turner BioSystem, CA), with a 10-second counting window. BSEP luciferase activity was reported relative to a control transfected Renilla luciferase (pRL-TK, Promega). All transfections were performed in triplicate and repeated in at least two separate sets of experiments (i.e. at least six individual data points for each experimental transient transfection).

Protein preparation. Whole cell lysates were prepared from cells suspended in 25 mM Tris-HCI (pH 7.8), 0.5 mM EDTA, and protease inhibitors (Roche, Nutley, NJ). Cells were lysed by four cycles of freezing and thawing, followed by centrifugation at 16,000 × g at 4°C for 15 min (21). Cellular membranes were obtained by centrifugation of whole cell lysate at 3,000 × g for 10 min at 4°C, followed by a second spin of the supernatant at 30,000 × g for 20 min (22). Preparation of nuclear and cytoplasmic proteins was performed using NE-PER (Pierce, Rockford, IL) according to manufacturer's protocol.

Protein immunoprecipitation. Protein immunoprecipitation was performed as described previously (23). Briefly, cytoplasmic and nuclear proteins prepared from cultured cells were centrifuged for 30 min at 10,000 × g, followed by preclearing the supernatants with 50 μL activated, quenched Sepharose by gently shaking for 1 h at 4°C and centrifugation for 5 min at 200 × g. The supernatants were mixed at 40:1 ratio with a 1:1 slurry of antibody-sepharose/Tris saline albumin (TSA) solution (0.14 M NaCl. 0.025% NaH3, 0.01 M Tris, pH 8.0, and 0.01% albumin), and gently shaken for 1 h at 4°C. The mixtures were washed three times with 0.1% Triton X-100 in TSA solution containing 0.01% albumin and 0.05 M Tris, pH 6.8. Fifty microliters of protein sample loading buffer without 2-mercaptoethanol were added to the Sepharose pellets and heated for 5 min at 100°C. After microcentrifugation for 5 s, the supernatant were analyzed by Western blot analysis.

Western Blot analysis. Total cellular membrane, nuclear, cytoplasmic, or immunoprecipitated proteins were separated by electrophoresis in 12% SDS–polyacrylamide gels overlaid with 4% of acryl amide stacking gels. The proteins were transferred to Hybond-C nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ), followed by blocking of nitrocellulose blots overnight at 4°C with 5% (wt/vol) nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (Sigma Chemical Co., St. Louis, MO). Antibody was added, and probing was conducted with gentle shaking at 4°C overnight. Antibody recognized bands were detected with the secondary antibody conjugated with horseradish peroxidase (Amersham), followed by chemiluminescent detection using ECL Western blotting detection reagents and analysis system (Amersham). Sample loading was examined by probing the same membrane with anti-Na+/K+-ATPase (membrane proteins), anti-actin (total and cytoplasmic proteins), or anti-histone H1 antibody (nuclear proteins).

Statistical analysis. Unless otherwise indicated, data are presented as mean values ± SD. Statistical analyses were performed using InStat Software (GraphPad Software Inc., San Diego, CA). For comparison of two values, t test was used assuming equal variance. For multiple comparisons, ANOVA was used. Differences were considered statistically significant at $p < 0.05$.

RESULTS

BSEP promoter activity in HepG2 cells. Initial investigations of the effects of FIC1 silencing were performed in HepG2 cells, which, unlike human hepatocytes, are readily available (Fig. 1). Cells were cultured in 0.5% CTFCS to permit analysis of the effect of the FXR ligands, CDCA and GW4064. Basal activity of the promoter was enhanced by the addition of CDCA or GW4064 ($p < 0.0001$ for control in 0.5% CTFCS versus either CDCA or GW4064). FIC1 silencing reduced promoter activity in the presence of these ligands, but not in their absence ($p > 0.05$ for control in 0.5% CTFCS versus siFIC1 in 0.5% CTFCS as opposed to $p < 0.001$ for the effects of siFIC1 with either CDCA or GW4064). Silencing of both FIC1 and FXR led to a synergistic repression in BSEP promoter activity in the presence or absence of FXR ligand ($p < 0.01$ for control in 0.5% CTFCS versus siFIC1/siFXR in 0.5% CTFCS and siFIC1/siFXR in 0.5% CTFCS versus either CDCA or GW4064). Treatment of the HepG2 cells with PKCζ pseudosubstrate inhibitor blocked FXR ligand-mediated activation of the BSEP promoter ($p > 0.05$ for 0.5% CTFCS, CDCA or GW4064).

Analysis of FIC1 silencing in human hepatocytes. FIC1 silencing in human hepatocytes led to a marked reduction in human BSEP promoter activity (control 913 ± 134; siFIC1 262 ± 30; siscrambled 952 ± 84, $p < 0.001$ for siFIC1 versus either control or siscrambled, relative light units corrected for Renilla luciferase transfection control, mean ± SD, n = 9 for each group, with three measurements each from three separate

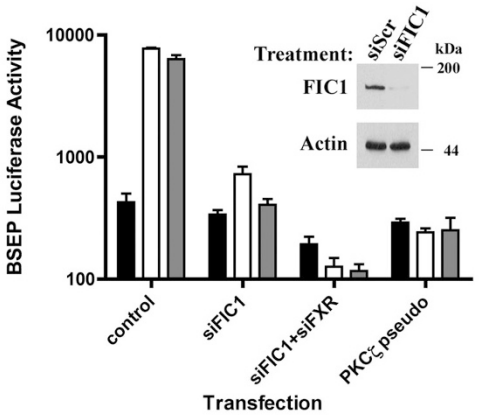

Figure 1. BSEP promoter analysis in HepG2 cells. HepG2 cells were cultured in 0.5% charcoal-treated FCS that was supplemented with 100 μM chenodeoxycholic acid (CDCA) or 10 μM GW 4064 as FXR ligands. FIC1 and/or FXR were silenced as indicated, or PKCζ was inhibited using a pseudosubstrate. Human BSEP promoter activity was assessed using a luciferase reporter. Bars represent the mean of nine measurements in three separate experiments. Error bars are SDs. The y axis is a logarithmic scale with BSEP luciferase reporter activity reported relative to a transfection efficiency control Renilla luciferase. Inset: western analysis of FIC1 silencing in HepG2 cells. ■ = 0.5% charcoal-treated FCS, □ = 100 μM CDCA, ▒ = 10 μM GW4064.

human hepatocyte preparations). This reduction in promoter activity could be correlated with a significant reduction in BSEP protein expression in human hepatocytes (siscrambled 44.2 ± 6.9 versus siFIC1 3.8 ± 0.6, densitometry units, n = 3, $p < 0.001$; Fig. 2). FIC1 silencing led to abrogation of FXR mediated signaling that was associated with a reduction in phosphorylation of FXR and a suggestion of redistribution of its localization to the cytoplasm (Figs. 3A and B).

Analysis of PKCζ on FXR signaling. PKCζ overexpression was associated with a marked induction of human BSEP promoter activity ($p < 0.001$ for wt PKCζ versus control),

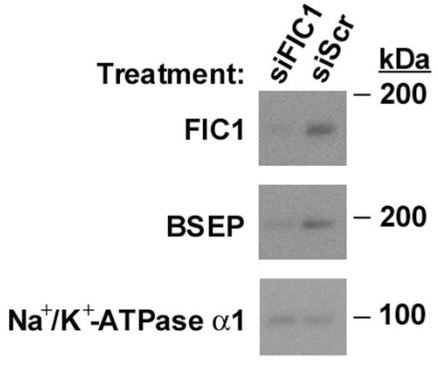

Figure 2. Western Blot analysis of the effect of FIC1 silencing on BSEP protein expression. Human hepatocytes were treated with siFIC1 or siscrambled, membrane proteins were prepared, and 20 μg of those proteins were probed with antibodies directed against FIC1, BSEP, and Na+/K+-ATPase (membrane loading control). FIC1 silencing is efficient (siscrambled 43.1 ± 13.7 vs siFIC1 1.4 ± 1.5 densitometry units, n = 3, $p < 0.001$) and associated with a reduction in BSEP protein expression (siscrambled 44.2 ± 6.9 vs siFIC1 3.8 ± 0.6, densitometry units, n = 3, $p < 0.001$). Equivalent loading is indicated by the signal for Na+/K+-ATPase. This blot is representative of three separate experiments with different human hepatocyte preparations.

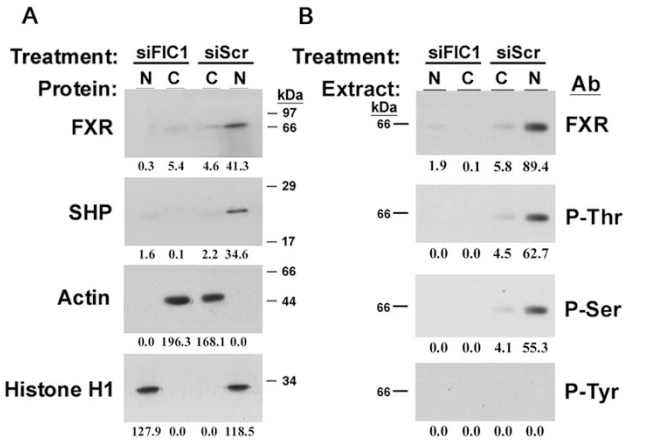

Figure 3. Western Blot analysis of the effect of FIC1 silencing on FXR. A, Human hepatocytes were treated with siFIC1 or siscrambled, 50 μg of cytoplasmic and 2.5 μg of nuclear proteins were prepared and probed with antibodies directed against FXR, SHP, and the loading controls actin (cytoplasmic) and Histone H1 (nuclear). FIC1 silencing leads to an overall reduction in FXR and SHP protein abundance with a redistribution of the FXR from a predominant nuclear to cytoplasmic localization in the FIC1 siRNA-treated cells. B, FXR was immunoprecipitated from the same protein extracts and then probed with general antibodies against phosphorylated (P-) threonine (Thr), serine (Ser), or tyrosine (Tyr). In the scrambled antisense control treated cells, FXR is phosphorylated on Thr and Ser but not Tyr. Phosphorylation is not detectable after siFIC1 treatment. In A and B, densitometry of specific bands is shown below the band.

whereas various means of reducing PKCζ expression [(silencing, pseudosubstrate inhibition, or dominant negative protein expression), $p < 0.001$ for comparison of each treatment relative with control] reduced BSEP promoter activity (Fig. 4A). The effects of changes in PKCζ on BSEP promoter activity were dependent on the presence of FXR ligand (Fig. 4B). When PKCζ was silenced, addition of the FXR ligands CDCA or GW4064 did not yield a significant increase in BSEP promoter activity ($p > 0.05$ for all comparisons for siPKCζ). In contrast, the BSEP promoter activity in the scrambled antisense control cells or those cells treated with wtPKCz was significantly enhanced with the addition of either 50 μM CDCA or in a dose-dependent fashion in response to either 0.2 or 1.0 μM GW4064 ($p < 0.01$ for all relevant comparisons). Ursodeoxycholic acid, which is not an FXR ligand, had no effect on BSEP promoter activity.

The findings from the promoter analyses were then confirmed by western blot analysis of human hepatocytes. PKCζ loss-of-function was associated with a reduction in BSEP protein expression, whereas gain-of-function resulted in its upregulation (Fig. 5, BSEP: control 15.9 ± 5.3, siPKCζ 1.3 ± 1.2, wt PKCζ 126.8 ± 2.0 densitometry units, $p < 0.01$ for all comparisons, FXR: control 13.9 ± 9.8, siPKCζ 1.1 ± 0.7, wt PKCζ 74.1 ± 50.6 densitometry units, $p > 0.05$ for all comparisons). Reducing PKCζ activity led to a redistribution of FXR from the nucleus to the cytoplasm and a reduction in both FXR and SHP protein levels (Fig. 6).

DISCUSSION

These studies have confirmed our previous investigations in enterocytes and Chinese hamster ovary cells and are consis-

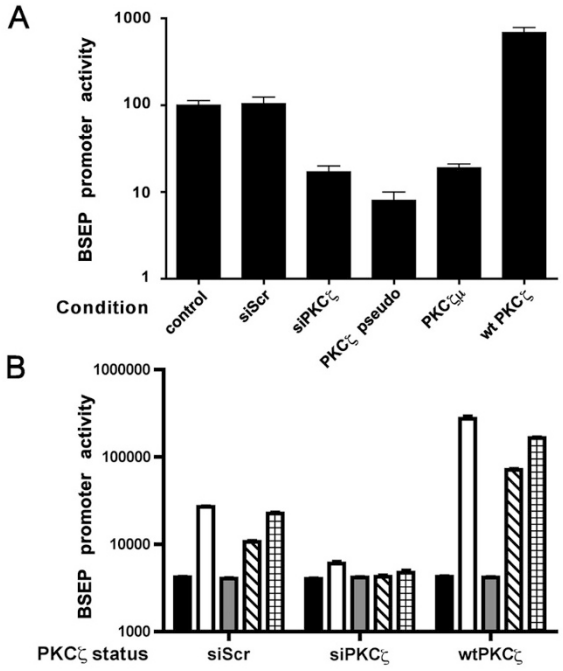

Figure 4. BSEP promoter analysis in human hepatocytes. A, Analysis in media supplemented with FCS. Freshly isolated human hepatocytes were cultured in media supplemented with 10% FCS. PKCζ activity was induced by overexpression of a wild type construct (wt PKCζ) or reduced by silencing (siPKCζ), pseudosubstrate inhibition (PKCζ pseudo), or by overexpression of a dominant negative protein (PKC$\zeta\mu$). Bars represent the mean of nine measurements in three separate experiments. B, Analysis of the effect of FXR ligand. Freshly isolated human hepatocytes were cultured in media supplemented with 0.5% charcoal-treated FCS that was supplemented with the following compounds to assess the dependence on the availability of FXR ligand: 50 μM CDCA, 50 μM UDCA, 0.2 μM GW4064, or 1.0 μM GW4064. PKCζ activity was induced by overexpression of a wild type construct (wt PKCζ) or reduced by silencing (siPKCζ). Human BSEP promoter activity was assessed using a luciferase reporter and are reported relative to control, set at 100%. Bars represent the mean of six measurements in two separate experiments. In both sets of studies, human BSEP promoter activity was assessed using a luciferase reporter and are reported relative to control, set at 100%, error bars are SDs, and the y axis is a logarithmic scale. ■ = bile acid free, □ = 50 μM CDCA, ▒ = 50 μM UDCA, ▨ = 0.2 μM GW4064, ▦ = 1.0 μM GW4064.

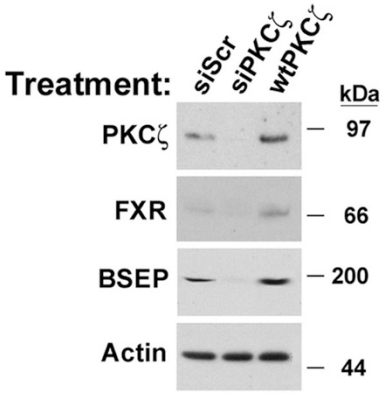

Figure 5. Western analysis of the effect of PKCζ on FXR and BSEP protein expression. Human hepatocytes were treated with either siPKCζ or a wild type PKCζ (wtPKCζ) expression construct and were compared with a control group of cells treated with a scrambled antisense siRNA (siScr). Fifty microgram of cellular homogenate proteins were sequentially probed for PKCζ, FXR, BSEP, and actin by western blot. This blot is representative of three separate experiments with different human hepatocyte preparations.

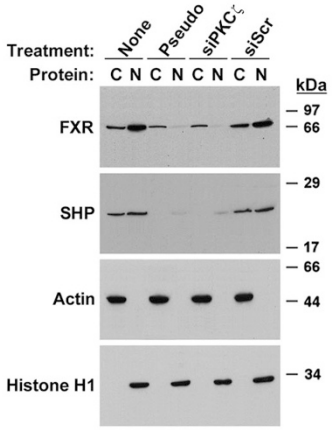

Figure 6. Western analysis of the effect of PKCζ inhibition on cellular localization of FXR and FXR-mediated signaling. PKCζ activity was reduced in human hepatocytes by either treatment with 100 μM pseudosubstrate inhibitor (Pseudo) or siRNA-mediated silencing (siPKCζ). Fifty microgram of cytoplasmic or 2.5 μg of nuclear proteins was sequentially probed for FXR, SHP, actin, and Histone H1. The latter two acting as cytoplasmic and nuclear protein loading controls, respectively.

tent with the recent findings from laboratories in Madrid and Tokyo using HepG2 cells (6,10,14,15). FIC1 loss-of-function in the setting of human disease results in diminished FXR signaling in ileum as assessed by Northern analysis (10). This phenomenon can be modeled in the human colon cancer cell line, Caco-2, when endogenous FIC1 is silenced using siRNA (10). The converse effect is observed with FIC1 gain-of-function in a Chinese hamster ovary cell line that lacks endogenous FIC1 protein expression (6). Of interest, FIC1 expression constructs with mutations described in benign recurrent intrahepatic cholestasis drive FXR activation at a level intermediate between wild type FIC1 and FIC1 mutants associated with severe disease (6). The current studies extend the observations from a hepatoblastoma-based cell line to freshly isolated human hepatocytes, which may be more relevant to human physiology despite the fact that these cells are not polarized (14,15).

It had been surmised from basic principles that PKCζ might mediate the signaling by FIC1. Atypical PKCs can be activated by phosphatidyl serine independent of calcium or diacylglycerol (24,25). In light of the proposed phosphatidylserine flipping properties of FIC1 and the precedent of PKCs as signal transduction molecules, PKCζ is an interesting candidate for mediation of the effect of FIC1 on FXR. Previous PKCζ loss-of-function analyses supported this hypothesis; in the absence of PKCζ, FIC1 mediated effects on FXR were lost (6). These studies are the first to demonstrate that PKCζ activity correlates with BSEP expression, both in the setting of loss- and gain-of-function. PKCζ-mediated changes in BSEP expression correlate with alterations in the expression and cellular localization of FXR. Diminished PKCζ activity correlates with reduced FXR expression, which is anomalously redistributed to the cytoplasm rather than the nucleus. In prior studies, we have shown that this change is related to the phosphorylation status of key threonine residues in FXR (6). The exact relationship between FIC1 and PKCζ activity re-

quires further investigation. In particular, it is not known whether there is a direct effect with activation of PKCζ by FIC1 or whether additional molecules transduce the signal between FIC1 and PKCζ. PKCζ has already been shown to influence the expression of another bile acid transport protein, although by a distinctly different mechanism (26,27).

Our findings are not entirely consistent with investigations of the mouse model of FIC1 disease or a recently published report of similar studies using human hepatocytes (16,28,29). Inconsistencies in the representation of human disease in mouse models are well described. The BSEP knock-out mouse in an unperturbed state has a minimal phenotype and does not display the type of liver disease seen in humans (30). This has been ascribed by some as the result of differences in bile acid homeostasis between mice and humans. In the initial description of the Byler mouse, hepatic and ileal gene expression was examined in wild type and Byler mice fed either a control diet or one enriched in cholic acid (28). Cholate feeding in Byler mice led to a 2-fold reduction in FXR, which was not seen in wild-type mice, suggesting an abnormality in FXR signaling. In another series of experiments, SHP expression was reduced in the liver of Byler mice and was upregulated after cholic acid feeding in Byler but not control mice (29). Other findings did not indicate a direct link between FIC1 and FXR. In light of the complexities of bile acid homeostasis in vivo, it can be difficult in animal models to understand a specific signaling pathway; hence, the potential power of a reductionist approaches using cells in culture. Cai et al. have performed experiments similar to those described in this work using a more complex cell culture system and adenoviral-mediated gene silencing. These very different technical approaches have the potential to explain some of the differences that have been observed in their studies compared with ours and those of others (16). Direct examination of FXR-mediated effects on promoter function was not performed in those analyses.

These studies support the hypothesis that FIC1 enhances the activity of FXR and its downstream targets via a signaling pathway involving PKCζ. It is clear that additional studies of the molecular mechanisms of FIC1 disease are needed. Continued analyses of the molecular biology of FIC1 function will inform future studies of disease (31). Novel approaches to the translational investigation of FIC1 disease using human tissues are also needed. Future studies of human liver will need to control for the potential nonspecific effects of progressive cholestatic liver disease, a daunting task.

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