2013

Mechanistic insights into isoform-dependent and species-specific regulation of bile salt export pump by farnesoid X receptor

Xiulong Song
Yuan Chen

See next page for additional authors

Follow this and additional works at: https://digitalcommons.uri.edu/bps_facpubs

Terms of Use
All rights reserved under copyright.
Mechanistic insights into isoform-dependent and species-specific regulation of bile salt export pump by farnesoid X receptor

Xiulong Song, Yuan Chen, Leila Valanejad, Rajani Kaimal, Bingfang Yan, Matthew Stoner, and Ruitang Deng

Department of Biomedical and Pharmaceutical Sciences, Center for Pharmacogenomics and Molecular Therapy, University of Rhode Island, Kingston, RI 02881

Abstract Expression of bile salt export pump (BSEP) is regulated by the bile acid/farnesoid X receptor (FXR) signaling pathway. Two FXR isoforms, FXRα1 and FXRα2, are predominately expressed in human liver. We previously showed that human BSEP was isoform-dependently regulated by FXR and diminished with altered expression of FXRα1 and FXRα2 in patients with hepatocellular carcinoma. In this study, we demonstrate that FXRα1 and FXRα2 regulate human BSEP through two distinct FXR-responsive elements (FXRE): IR1α and IR1β. As the predominant regulator, FXRα2 potently transactivated human BSEP through IR1α, while FXRα1 weakly transactivated human BSEP through a newly identified IR1β. Relative expression of FXRα1 and FXRα2 affected human BSEP expression in vitro and in vivo. Electrophoretic mobility shift and chromatin immunoprecipitation assays confirmed the binding and recruitment of FXRα1 and FXRα2 to IR1β and IR1α. Sequence analysis concluded that IR1β was completely conserved among species, whereas IR1α exhibited apparent differences across species. Sequence variations in IR1α were responsible for the observed species differences in BSEP transactivation by FXRα1 and FXRα2. In conclusion, FXR regulates BSEP in an isoform-dependent and species-specific manner through two distinct FXREs, and alteration of relative FXR isoform expression may be a potential mechanism for FXR to precisely regulate human BSEP in response to various physiological and pathological conditions. Song, X., Y. Chen, L. Valanejad, R. Kaimal, B. Yan, M. Stoner, and R. Deng. Mechanistic insights into isoform-dependent and species-specific regulation of bile salt export pump by farnesoid X receptor. J. Lipid Res. 2013. 54: 3030–3044.

Supplementary key words bile acid transporter • canalicular secretion • gene transcription

Bile acid homeostasis is achieved through an enterohepatic circulation. Canalicular secretion of bile acids through bile salt export pump (BSEP, ABCB11) is the rate-limiting step in the circulation (1, 2). Modulation of BSEP expression or function by inherited or acquired factors has profound impact on biliary and intrahepatic bile acid levels. Indeed, impairment of BSEP function or expression has been directly linked to such diseases as progressive familial intrahepatic cholestasis type 2 (5–6), benign recurrent intrahepatic cholestasis (7–9), intrahepatic cholestasis of pregnancy (10, 11), drug-induced cholestasis (12), and liver cancer (6, 13, 14).

BSEP expression is coordinately regulated by distinct but related transactivation pathways (15–19), notably the bile acids/farnesoid X receptor (FXR, NR1H4) signaling pathway (15, 16). Activation of FXR by bile acids strongly induces BSEP expression in vitro and in vivo (15, 16). Such feed-forward regulation of BSEP by bile acid/FXR is considered a major mechanism for preventing excessive accumulation of toxic bile acids in hepatocytes. Two FXR genes, FXRα and FXRβ, have been identified (20–22). FXRα is functional in all the species tested, while FXRβ is a pseudogene in humans (22). Four isoforms of FXRα (FXRα1–4) have been identified as a result of alternative promoter and splicing (23, 24). Isoforms FXRα1 and FXRα2 are predominantly expressed in the liver and adrenal glands, whereas isoforms FXRα3 and FXRα4 are primarily expressed in the kidneys and intestines in humans (23).

Abbreviations: BSEP, bile salt export pump; CA, cholic acid; CDCA, chenodeoxycholic acid; ChIP, chromatin immunoprecipitation; DCA, deoxycholic acid; DIC, differential interference contrast; EMSA, electrophoretic mobility shift assay; ER2, everted repeat separated by two nucleotides; FXR, farnesoid X receptor; FXRE, FXR-responsive element; FXREc, FXRE consensus; GDC, glycodeoxycholic acid; GUG, guggulsterone; HCC, hepatocellular carcinoma; IR1, inverted repeat with one nucleotide space; LCA, lithocholic acid; NR1H4, nuclear receptor subfamily 1, group H, member 4; UDCA, ursodeoxycholic acid; wt, wild-type.

1To whom correspondence should be addressed.
e-mail: dengr@mail.uri.edu

This work was supported by the National Institutes of Health Grants R01 DK087755, R01 GM-61988 (B.Y.), and R01 ES-07965 (B.Y.). The RI-INBRE Core Facility is supported by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under Grant 8 P20 GM-103430-12.

Manuscript received 29 March 2013 and in revised form 18 August 2013.

Published, JLR Papers in Press, September 3, 2013
DOI 10.1194/jlr.M038323

Copyright © 2013 by the American Society for Biochemistry and Molecular Biology, Inc.

This article is available online at http://www.jlr.org
We have recently showed that human BSEP expression was severely diminished in patients with hepatocellular carcinoma (HCC) and that such decreased expression of BSEP correlated with altered relative expression of FXR1a and FXR1b (25). Similar to human BSEP, FXR1b showed more potent activity than FXR1a in transactivating other hepatic FXR targets, including human syndecan-1 and fibrinogens (26, 27), whereas mouse bsep and small heterodimeric partner (SHP) were regulated by FXR1a and FXR1b to similar degrees (24). Currently, the underlying mechanisms for such isoform-dependent and species-specific regulation of BSEP by FXR are not understood.

In this study, we demonstrate that FXR1a and FXR1b transactivated human BSEP through two distinct FXR responsive elements (FXRE) that are inverted repeats spaced by one nucleotide (IR1): IR1b and IR1a, respectively. In addition to total FXR expression levels, relative expression of FXR1a and FXR1b affected human BSEP expression in vitro and in vivo. Further study revealed that sequence variations in IR1a were responsible for the observed species difference in transactivation of BSEP by FXR1a and FXR1b.

**EXPERIMENTAL PROCEDURES**

**Reagents and suppliers**

Chemicals and reagents for cloning, site-directed mutagenesis, polymerase chain reaction (PCR), gel-shift assays, cell cultures, transfection, and luciferase assays were described previously (28).

**Plasmid constructs**

Human, mouse, and rat bsep promoter reporters and mutants, including pmBSEP(-2.6kb), pmBSEP(-2.6kb), prBSEP(-2kb), phBSEP(-125b), and prBSEP(-2.6kb)-IR1a-Mut, were prepared previously (18, 28, 29). Human pTK-3xIR1a, mouse/rat pTK-3xIR1b, and pTK-3xIR1b reporters were constructed by cloning three copies of the corresponding element into pTK-Luc vector. Element reporters pGL3/p-3xIR1a and pGL3/p-2xIR2/IR1a were prepared by cloning two copies of IR1a and ER2/IR1a element in human BSEP promoter into the pGL3/p vector, respectively. Element reporters, including eElement 1-6, were generated by cloning the corresponding element into the pGL3/p vector. Expression plasmids for human nuclear receptors FXR1a and FXR1b were kindly provided by Dr. Matthew Stoner and Dr. David Mangelsdorf (University of Texas Southwestern Medical Center), respectively.

**Liver samples**

Snap-frozen healthy human liver samples were obtained through the Mid-Atlantic Division of the Cooperative Human Tissue Network (CHTN) (University of Virginia). The protocol for using human tissues was approved by the Institutional Review Board (IRB) at the University of Rhode Island. Tissue Network (CHTN) (University of Virginia). The protocol for using human tissues was approved by the Institutional Review Board (IRB) at the University of Rhode Island.

**Site-directed mutagenesis**

Mutations were introduced into the templates using a Quick-Change site-directed mutagenesis kit according to the manufacturer’s manual (Stratagene) (29). Human BSEP promoter mutants prBSEP(-2kb)-IR1b-Mut, prBSEP(-2kb)IR1b-Mut, and prBSEP(-2kb)-IR2-Mut were prepared by mutating the IR1b, IR1a and IR1b, and half ER2 (everted repeat separated by two nucleotides) sites using prBSEP(-2kb) as a template. Six, four, and two nucleotides were mutated in IR1a (GAGGCGTGAATTTC), IR1b (ATGGCCCTTCTACT), and the half ER2 site (AACCCT), respectively (mutated nucleotides are underlined). Rat bsep promoter mutants prBSEP(-2kb)-IR1a-Mut, prBSEP(-2kb)-IR1b-Mut, prBSEP(-2kb)-IR1a-Mut, and prBSEP(-2kb)-ER2-Mut were prepared by mutating the IR1a, IR1b, IR1a and IR1b, and the half ER2 site using prBSEP(-2kb) as templates. Three, four, and three nucleotides were mutated in IR1a (GGGGACATTGC), IR1b (ATGGCCCTTCTACT), and the half ER2 site (AAAAACT), respectively. Replacement of human IR1a (gIR1a) with mouse/rat IR1a (m/rIR1a) was achieved through mutagenesis using phBSEP(-2.6kb) as the template, resulting in a chimeraic reporter phBSEP(-2.6kb)-m/rIR1a. Chimeric reporter prBSEP(-2kb)-IR1a was made through mutagenesis by replacing the mouse/rat IR1a (m/rIR1a) with the human IR1a (hIR1a) using prBSEP(-2kb) as the template. All the mutants were subjected to sequence confirmation.

**Immunohistofluorescence**

Normal human liver tissue sections embedded in paraffin (ab4348) were obtained from Abcam (Cambridge, MA). Deparaffinization, antigen retrieval, and immune staining of the sections were carried out basically according to the protocol recommended by the manufacturers. Nonspecific binding was blocked by using 5% BSA in 1× TBS containing 0.1% TX-100 for 2 h on shaker at room temperature. Three types of primary antibodies (Abs) were applied to individual slides, including rabbit anti-FXRα1-specific Abs (custom-made by the NeoBioLab, Woburn, MA), rabbit anti-FXR Abs (sc-13063), and rabbit IgG (sc-2027, Santa Cruz Biotechnology) as negative controls. AlexaFluor 594 goat anti-rabbit Abs (A11012, Invitrogen) was used as the secondary Abs. The sections were then mounted under a glass cover slip using VectaShield mounting medium containing 1.5 μg/ml DAPI (H-1500, Vector Laboratories, Burlingame, CA) as a nuclei counterstaining. Images were captured under a confocal microscope at a magnification of 40× (Zeiss AxioImager M2 Imaging System) with fluorescent and differential interference contrast (DIC) settings.

**Reporter luciferase assay**

The reporter luciferase activity assays were carried out using the human hepatoma HuH 7 cells and rat hepatocellular carcinoma Ccl7 cells with either FXR1a or FXR2 expression plasmid, followed by treatment of the transfected cells with vehicle DMSO (0.1%) or CDCA (10 μM) for 30 h. Reporter transactivation levels as luciferase activities were detected with a dual-luciferase reporter assay system. The firefly luminescence was normalized based on the Renilla luminescence signal. The data are presented as mean ± SD of at least three separate experiments, *P<0.05 between DMSO and CDCA-treated cells (Student’s t-test).

---

**Fig. 1.** Transactivation of BSEP by FXRα1 and FXRα2. Human, mouse, and rat bsep promoter reporters phBSEP(-2.6kb), pmBSEP(-2.6kb), and prBSEP(-2kb) were cotransfected into human hepatoma Huh 7 cells with either FXRα1 or FXRα2 expression plasmid, followed by treatment of the transfected cells with vehicle DMSO (0.1%) or CDCA (10 μM) for 30 h. Reporter transactivation levels as luciferase activities were detected with a dual-luciferase reporter assay system. The firefly luminescence was normalized based on the Renilla luminescence signal. The data are presented as mean ± SD of at least three separate experiments, *P<0.05 between DMSO and CDCA-treated cells (Student’s t-test).
(HCC) L0 cells (kindly provided by Dr. Jonathan Reicher, Brown University) in a 24-well plate as described previously (28).

Electrophoretic mobility shift assays

Nuclear extracts of Huh 7 cells transfected with FXRα1 or FXRα2 were prepared using the NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce). The electrophoretic mobility shift assays (EMSA) were performed using the LightShift Chemiluminescent EMSA kit (Pierce) as described (17). Supershift assays were carried out using polyclonal Abs against human FXR (sc-13063, Santa Cruz Biotechnologies).

Chromatin immunoprecipitation assays

Huh 7 cells seeded in 6-well plates were cotransfected with 400 ng of either FXRα1 or FXRα2 and either 400 ng phBSEP (-2.6kb), phBSEP(-2.6kb)-IR1a-Mut, phBSEP(-2.6kb)-IR1b-Mut, or phBSEP(-2.6kb)-IR1ab-Mut. Sixteen hours posttransfection, cells were treated with 10 μM CDCA for 24 h. Chromatin preparation and immunoprecipitation (ChIP) assays were performed essentially as described previously (28). A test set of primers, BSEP(-260b) and pGL4-R(122), flanking IR1a and IR1b were used to amplify a 462 bp fragment encompassing sequence from nucleotides −260 to +80 in the BSEP promoter and 122 nucleotides in the luciferase gene. A negative control set of primers was included in the assays (28).

Living imaging with in vivo imaging system

Sixteen female CD-1 mice between ages 5 and 8 weeks were obtained from Charles River Laboratory (Wilmington, MA) and randomly divided into four groups. Each group of mice was

---

Fig. 2. The effects of relative expression levels of FXRα1 and FXRα2 on BSEP expression in human liver tissues and Huh 7 cells. (A) mRNA expression levels of BSEP and total FXR in healthy human liver tissues were determined by TaqMan real-time PCR and plotted against FXRα1/FXRα2 ratios using SigmaPlot version 11.0. The cycle time (Ct) values Cr24.92 for FXR and Cr27.12 for BSEP of one tissue sample were designated as a relative unit 1. (B) A series of mixtures of FXRα1 and FXRα2 plasmid DNA in various FXRα1/FXRα2 ratios (from 1.0, 1.2, 1.4, 1.6 to 1.8) with a constant amount of total FXR at 10, 25, or 50 ng were cotransfected with human BSEP promoter reporter in Huh 7 cells, followed by treatment of the transfected cells with CDCA for 30 h and subsequent detection of luciferase activity. FXRα1/FXRα2 ratios were plotted against total FXR and BSEP promoter transactivation as luciferase activities using SigmaPlot version 11.0. The left figure shows the level and position of each individual sample in the 3D diagram. The right figure is the smooth 3D model based on the data shown on the left.
Isoform and species-specific regulation of BSEP by FXR

Associated with dysregulation of BSEP in patients with HCC (25). In this study, we extended such finding by testing FXR isoform-mediated transactivation of BSEP from three species, including human, mouse, and rat. Consistent with our previous findings, FXRα2 exhibited much more potent activity in transactivating human BSEP than FXRα1 in the presence or absence of endogenous FXR agonist chenodeoxycholic acid (CDCA) (Fig. 1). In contrast, both mouse and rat bsep were transactivated by FXRα1 and FXRα2 in comparable levels (Fig. 1). The results demonstrated that human BSEP was differentially regulated by FXRα1 and FXRα2 with FXRα2 being the predominant regulator, whereas mouse and rat bsep were similarly transactivated by the two isoforms.

Relative FXR isoform and total FXR expression affected the expression levels of human BSEP

In our previous studies, it was shown that the relative expression levels of FXRα1 and FXRα2 correlated with BSEP expression (25). In this study, the comprehensive relationships among FXR isoforms, total FXR expression, and BSEP expression were further investigated. As shown in Fig. 2A, the expression levels of BSEP and total FXR in healthy human liver samples were plotted against FXRα1/FXRα2 ratios using SigmaPlot 3D program. Over 27- and 6-fold differences in BSEP and total FXR expression, respectively, were detected among the liver samples. Consistent with FXR’s transactivating role in BSEP regulation, BSEP expression increased along with increase in total FXR. However, the relationship between FXRα1/FXRα2 ratios and BSEP expression did not exhibit a single trend (Fig. 2A). At higher levels of total FXR, as expected, BSEP expression decreased as the FXRα1/FXRα2 ratios increased. However, at lower levels of total FXR, BSEP expression increased as the FXRα1/FXRα2 ratios increased. The data thus indicated that BSEP expression in human liver cDNA samples as templates, the relative FXRα1 and FXRα2 mRNA levels were determined by the semi-quantification methods described previously (25).

Statistical analysis

Student t-test was applied to pair-wise comparison to determine the statistical significance. Nonparametric Mann-Whitney test was used for pair-wise comparison for nonnormally distributed data. Values of 0.05 or less were considered significant.

RESULTS

Isoform-dependent and species-specific regulation of BSEP transactivation by FXR

Four FXRα isoforms (FXRα1–4) have been identified, with predominant expression of FXRα1 and FXRα2 in the liver (23, 24). Our previous study showed that human BSEP was regulated by FXR in an isoform-dependent manner and that alteration of isoform expression was associated with dysregulation of BSEP in patients with HCC (25). In this study, we extended such finding by testing FXR isoform-mediated transactivation of BSEP from three species, including human, mouse, and rat. Consistent with our previous findings, FXRα2 exhibited much more potent activity in transactivating human BSEP than FXRα1 in the presence or absence of endogenous FXR agonist chenodeoxycholic acid (CDCA) (Fig. 1). In contrast, both mouse and rat bsep were transactivated by FXRα1 and FXRα2 in comparable levels (Fig. 1). The results demonstrated that human BSEP was differentially regulated by FXRα1 and FXRα2 with FXRα2 being the predominant regulator, whereas mouse and rat bsep were similarly transactivated by the two isoforms.

Relative FXR isoform and total FXR expression affected the expression levels of human BSEP

In our previous studies, it was shown that the relative expression levels of FXRα1 and FXRα2 correlated with BSEP expression (25). In this study, the comprehensive relationships among FXR isoforms, total FXR expression, and BSEP expression were further investigated. As shown in Fig. 2A, the expression levels of BSEP and total FXR in healthy human liver samples were plotted against FXRα1/FXRα2 ratios using SigmaPlot 3D program. Over 27- and 6-fold differences in BSEP and total FXR expression, respectively, were detected among the liver samples. Consistent with FXR’s transactivating role in BSEP regulation, BSEP expression increased along with increase in total FXR. However, the relationship between FXRα1/FXRα2 ratios and BSEP expression did not exhibit a single trend (Fig. 2A). At higher levels of total FXR, as expected, BSEP expression decreased as the FXRα1/FXRα2 ratios increased. However, at lower levels of total FXR, BSEP expression increased as the FXRα1/FXRα2 ratios increased. The data thus indicated that BSEP expression in human liver cDNA samples as templates, the relative FXRα1 and FXRα2 mRNA levels were determined by the semi-quantification methods described previously (25).

Statistical analysis

Student t-test was applied to pair-wise comparison to determine the statistical significance. Nonparametric Mann-Whitney test was used for pair-wise comparison for nonnormally distributed data. Values of 0.05 or less were considered significant.

RESULTS

Isoform-dependent and species-specific regulation of BSEP transactivation by FXR

Four FXRα isoforms (FXRα1–4) have been identified, with predominant expression of FXRα1 and FXRα2 in the liver (23, 24). Our previous study showed that human BSEP was regulated by FXR in an isoform-dependent manner and that alteration of isoform expression was associated with dysregulation of BSEP in patients with HCC (25). In this study, we extended such finding by testing FXR isoform-mediated transactivation of BSEP from three species, including human, mouse, and rat. Consistent with our previous findings, FXRα2 exhibited much more potent activity in transactivating human BSEP than FXRα1 in the presence or absence of endogenous FXR agonist chenodeoxycholic acid (CDCA) (Fig. 1). In contrast, both mouse and rat bsep were transactivated by FXRα1 and FXRα2 in comparable levels (Fig. 1). The results demonstrated that human BSEP was differentially regulated by FXRα1 and FXRα2 with FXRα2 being the predominant regulator, whereas mouse and rat bsep were similarly transactivated by the two isoforms.

Relative FXR isoform and total FXR expression affected the expression levels of human BSEP

In our previous studies, it was shown that the relative expression levels of FXRα1 and FXRα2 correlated with BSEP expression (25). In this study, the comprehensive relationships among FXR isoforms, total FXR expression, and BSEP expression were further investigated. As shown in Fig. 2A, the expression levels of BSEP and total FXR in healthy human liver samples were plotted against FXRα1/FXRα2 ratios using SigmaPlot 3D program. Over 27- and 6-fold differences in BSEP and total FXR expression, respectively, were detected among the liver samples. Consistent with FXR’s transactivating role in BSEP regulation, BSEP expression increased along with increase in total FXR. However, the relationship between FXRα1/FXRα2 ratios and BSEP expression did not exhibit a single trend (Fig. 2A). At higher levels of total FXR, as expected, BSEP expression decreased as the FXRα1/FXRα2 ratios increased. However, at lower levels of total FXR, BSEP expression increased as the FXRα1/FXRα2 ratios increased. The data thus indicated that BSEP expression in human liver cDNA samples as templates, the relative FXRα1 and FXRα2 mRNA levels were determined by the semi-quantification methods described previously (25).

Statistical analysis

Student t-test was applied to pair-wise comparison to determine the statistical significance. Nonparametric Mann-Whitney test was used for pair-wise comparison for nonnormally distributed data. Values of 0.05 or less were considered significant.

RESULTS

Isoform-dependent and species-specific regulation of BSEP transactivation by FXR

Four FXRα isoforms (FXRα1–4) have been identified, with predominant expression of FXRα1 and FXRα2 in the liver (23, 24). Our previous study showed that human BSEP was regulated by FXR in an isoform-dependent manner and that alteration of isoform expression was associated with dysregulation of BSEP in patients with HCC (25). In this study, we extended such finding by testing FXR isoform-mediated transactivation of BSEP from three species, including human, mouse, and rat. Consistent with our previous findings, FXRα2 exhibited much more potent activity in transactivating human BSEP than FXRα1 in the presence or absence of endogenous FXR agonist chenodeoxycholic acid (CDCA) (Fig. 1). In contrast, both mouse and rat bsep were transactivated by FXRα1 and FXRα2 in comparable levels (Fig. 1). The results demonstrated that human BSEP was differentially regulated by FXRα1 and FXRα2 with FXRα2 being the predominant regulator, whereas mouse and rat bsep were similarly transactivated by the two isoforms.

Relative FXR isoform and total FXR expression affected the expression levels of human BSEP

In our previous studies, it was shown that the relative expression levels of FXRα1 and FXRα2 correlated with BSEP expression (25). In this study, the comprehensive relationships among FXR isoforms, total FXR expression, and BSEP expression were further investigated. As shown in Fig. 2A, the expression levels of BSEP and total FXR in healthy human liver samples were plotted against FXRα1/FXRα2 ratios using SigmaPlot 3D program. Over 27- and 6-fold differences in BSEP and total FXR expression, respectively, were detected among the liver samples. Consistent with FXR’s transactivating role in BSEP regulation, BSEP expression increased along with increase in total FXR. However, the relationship between FXRα1/FXRα2 ratios and BSEP expression did not exhibit a single trend (Fig. 2A). At higher levels of total FXR, as expected, BSEP expression decreased as the FXRα1/FXRα2 ratios increased. However, at lower levels of total FXR, BSEP expression increased as the FXRα1/FXRα2 ratios increased. The data thus indicated that BSEP expression in human
livers was dictated by the FXRα1/FXRα2 ratios as well as by total FXR expression levels.

To directly evaluate the effect of FXRα1/FXRα2 ratios at various total FXR levels on BSEP transactivation in vitro, a series of mixtures of FXRα1 and FXRα2 plasmid DNA in various ratios (from 1:1 to 1.8:1) at various amounts of total FXR (10, 25, or 50 ng) were cotransfected with the human BSEP promoter reporter in Huh 7 cells, followed by treatment with CDCA. As shown in Fig. 2B, a relationship pattern comparable to human liver samples (Fig. 2A) was observed in transfected Huh 7 cells. BSEP transactivation gradually decreased as the FXRα1/FXRα2 ratios increased at a high level of total FXR plasmid DNA (50 ng) but increased at a low level of total FXR plasmid DNA (10 ng). The minor differences between the two data sets (Fig. 2) may reflect the fact that the sample size of human liver samples was relatively small. Taken together, the results demonstrated that the ratio of FXRα1/FXRα2 played an important role in determining human BSEP transactivation and that its effects were influenced by the total FXR expression levels.

Expression and localization of FXRα1 and total FXR protein in human liver tissue

Immunohistofluorescence was carried out to investigate the expression and localization of total FXR and FXRα1. In our attempt to generate FXR isoform-specific Abs, antiserum raised against FXRα1-specific peptide detected FXRα1 protein specifically, whereas antiserum raised against FXRα2-specific peptide recognized both FXRα2 and FXRα1 protein (data not shown) because there are only four exact amino acids inserted in FXRα1 compared with FXRα2. Therefore, FXRα1-specific Abs and Abs recognizing both FXRα1 and FXRα2 were used in the immunohistofluorescent assays. As shown in Fig. 3, minimal red fluorescent signals were detected with rabbit IgG as negative controls while FXRα1 and total FXR proteins were readily detected with FXRα1- and FXR-specific Abs. Both FXRα1 and total FXR proteins were predominantly present in the cytoplasm. As expected, relatively weaker signals were detected with FXRα1-specific Abs than with FXR-specific Abs.

Isoform-dependent transactivation of human BSEP by FXR was not due to a difference in ligand-mediated activation

In the previous experiments, the potent endogenous FXR agonist CDCA was used in evaluating human BSEP transactivation by FXR isoforms. To determine whether FXRα1- and FXRα2-mediated differential transactivation of human BSEP is related to specific FXR ligands, a series of FXR endogenous and exogenous ligands were tested for their ability to induce FXRα1- and FXRα2-mediated transactivation of human BSEP. The ligands tested include six endogenous bile acids, including cholic acid (CA), CDCA, deoxycholic acid (DCA), lithocholic acid (LCA), ursodeoxycholic acid (UDCA) and glycodeoxycholic acid (GDCA), a natural product guggulsterone, and two synthetic FXR agonists GW4064 and T0901317. As shown in Fig. 4A, all the FXR ligands exhibited similar FXR isoform-dependent transactivation of human BSEP regardless their agonistic potency. FXRα2 consistently transactivated human BSEP at a much higher level than FXRα1 with each of the ligands.

In contrast to human BSEP, both mouse and rat bsep promoters exhibited comparable transactivation activities by FXRα1 and FXRα2, with slightly higher levels with FXRα2 in the presence of CDCA in Huh 7 cells (Fig. 1). Considering the possibility that rat or mouse bsep may exhibit a different transactivation pattern by FXRα1 and FXRα2 in rat or mouse cells, rat bsep promoter reporter prBSEP(-2kb) was cotransfected with FXRα1 or FXRα2 in rat HCC L0 cells, followed by treatment with various FXR ligands. As shown in Fig. 4B, consistent with the data from Huh 7 cells, FXRα1 and FXRα2 exhibited similar transactivation levels with most FXR ligands, except for CDCA and LCA. FXRα2 transactivated rat bsep more potently than did FXRα1 with CDCA and LCA. However, it should be emphasized that the differences between FXRα2 and FXRα1 in transactivating human BSEP in Huh 7 cells were
Isoform and species-specific regulation of BSEP by FXR

Previous studies revealed that half nuclear receptor binding site and ER2 (everted repeat separated by two nucleotides) are commonly found adjacent to the IR1 (30). A half ER2 motif was identified immediately up-stream the IR1a (Fig. 5C). Inclusion of the motif in hER2/IR1a reporter significantly enhanced FXR/H9251-mediated trans-activation (Fig. 5C), indicating that the half ER2 motif is required for maximal transactivation of human BSEP by FXR/H9251. However, FXR/H9251 still failed to transactivate the hER2/IR1a reporter, suggesting that FXR/H9251 must regulate human BSEP through an additional FXRE.

FXR/H9251 transactivated human BSEP through IR1b, a newly identified FXRE.

To determine whether the potential additional FXRE is present in the minimal human BSEP promoter, the resultant phBSEP(-125b) reporter was cotransfected with FXRα1, followed by treatment with DMSO or CDCA. As shown in Fig. 6A, similar to the full-length promoter reporter phBSEP(-125b), FXRα1 significantly transactivated the minimal promoter reporter, indicating that the potential additional FXRE is located in the minimal promoter region.

Since no FXREs were predicted by bioinformatic analysis in the region, a series of element reporters covering the entire region were constructed and evaluated for their ability to respond to FXRα1 (Fig. 6B, C). Reporters pElement 1 and 4 exhibited significant induction of activity in response to CDCA in the presence of FXRα1, whereas no induction was detected with other reporters. The data thus mapped a new functional FXRE (named IR1b) in the overlapped region between pElement 1 and 4 at positions from much more striking than those with rat bsep in rat HCC L0 cells. FXRα2 was 27.1- or 18.9-fold more potent than FXRα1 in transacting human BSEP in Huh 7 cells in the presence of CDCA or LCA, whereas only 2.6- or 4.1-fold differences were observed between FXRα2 and FXRα1 in transactivating rat bsep in L0 cells with CDCA or LCA.

Taken together, the data indicated that the isoform-dependent transactivation of human BSEP by FXR was not due to a difference between FXRα1 and FXRα2 in ligand binding or ligand-mediated activation. On the other hand, FXRα1 and FXRα2 transactivated rat bsep comparably with most of the FXR ligands.

Isoform-dependent and species-specific transactivation of BSEP by FXR was due to a difference in FXRE in BSEP promoters

Early studies identified an FXRE (named IR1a) in the BSEP promoter (15, 16). To determine whether the isoform-dependent and species-specific transactivation of BSEP is due to a differential activation of the IR1a by FXRα1 and FXRα2, human and mouse/rat IR1a (hIR1a and m/rIR1a) reporters were constructed and tested for their ability to support FXRα1 or FXRα2 transactivation. It should be mentioned that the IR1a sequences are completely conserved in mouse and rat bsep promoters. As shown in Fig. 5A, FXRα2 significantly transactivated hIR1a reporter, whereas no transactivation was detected in cells transfected with FXRα1. Conversely, both FXRα1 and FXRα2 strongly transactivated m/rIR1a reporter (Fig. 5B). The results thus demonstrated that hIR1a only supported FXRα2 transactivation, while m/rIR1a was capable of mediating both FXRα1 and FXRα2 transactivation.

Previous studies revealed that half nuclear receptor binding site and ER2 (everted repeat separated by two nucleotides) are commonly found adjacent to the IR1 (30). A half ER2 motif was identified immediately up-stream the IR1a (Fig. 5C). Inclusion of the motif in hER2/IR1a reporter significantly enhanced FXRα2-mediated transactivation (Fig. 5C), indicating that the half ER2 motif is required for maximal transactivation of human BSEP by FXRα2. However, FXRα1 still failed to transactivate the hER2/IR1a reporter, suggesting that FXRα1 must regulate human BSEP through an additional FXRE.

FXRα1 transactivated human BSEP through IR1b, a newly identified FXRE.
Fig. 6. FXR\textsubscript{1} transactivated human BSEP through a newly identified FXRE, IR1b. (A) Full-length and minimal human BSEP promoter reporters pBSEP(-2.6kb) and pBSEP(-125b) were cotransfected with FXR\textsubscript{1} in Huh 7 cells. Sixteen hours posttransfection, cells were treated with DMSO (0.1%) and CDCA (10 \textmu M) for 30 h, followed by detection luciferase activities with dual-luciferase reporter assay system. The data are presented as mean ± SD of at least three repeated experiments. (B) A diagram showing the positions of the six elements in the minimal promoter region. (C) The six elements were cloned into the pGL3/p-Luc vector, resulting in six element reporters, pElement 1 to 6. Activation of the six element reporter by FXR\textsubscript{1} in the presence of CDCA (10 \textmu M) was detected by the dual-luciferase reporter assay system. The data are presented as mean ± SD of at least three repeated experiments. *P < 0.05 between DMSO and CDCA-treated cells (Student \textit{t}-test). (D) The location and sequence of the newly identified IR1b are shown in the upper panel. Transactivation of hIR1a and IR1b reporters by FXR\textsubscript{1} are presented in the lower panel. Reporters pTK-3xhIR1a and pTK-3xIR1b were cotransfected into Huh 7 cells with FXR\textsubscript{1}, followed by treating the cells with DMSO (0.1%) or CDCA (10 \textmu M) for 30 h prior to detection of luciferase activities described in (A). *P < 0.05 between DMSO and CDCA-treated cells (Student \textit{t}-test).

Mutational analysis of IR1a and IR1b in supporting FXR\textsubscript{1}- and FXR\textsubscript{2}-mediated transactivation of human and rat bsep

To determine the relative contribution of IR1a, IR1b, and the half ER2 sites to the transactivation of human and rat bsep by FXR\textsubscript{1} and FXR\textsubscript{2}, mutational analyses were performed in those elements. Double mutation in IR1a and IR1b was also introduced.

For human BSEP, similar to the IR1a and IR1b double mutant, IR1b mutant completely lost its ability to respond to FXR\textsubscript{1} (Fig. 7A), suggesting that FXR\textsubscript{1} regulates human BSEP through IR1b. Although mutation in IR1a decreased FXR\textsubscript{1}-mediated transactivation, significant induction remained (Fig. 7A). More significantly, the fold induction by CDCA over DMSO was comparable between IR1a mutant and wild-type (wt) (13.1 versus 11.3), indicating that IR1a is required for basal activity but not FXR\textsubscript{1}-mediated transactivation. Conversely, mutation in IR1a almost entirely abolished FXR\textsubscript{2}-mediated transactivation as did the double mutant (Fig. 7B), consistent with regulation of human BSEP by FXR\textsubscript{2} through IR1a. Similarly, the IR1b mutant retained the ability to respond to CDCA in the presence of FXR\textsubscript{2} with almost identical fold inductions as the wt (7.9 versus 7.4) (Fig. 7B). Mutation in the half ER2 motif markedly reduced FXR\textsubscript{2}- but not FXR\textsubscript{1}-mediated transactivation (Fig. 7A, B). Taken together, the data demonstrated that FXR\textsubscript{1} and FXR\textsubscript{2} transactivated human BSEP through IR1b and IR1a, respectively, and that the ER2 motif is required for maximal FXR\textsubscript{2} transactivation.

Similar mutational analyses were performed with rat bsep promoter to evaluate species differences. As shown in Fig. 7C, mutation in IR1a or IR1b resulted in a decrease in FXR\textsubscript{1}-mediated transactivation. However, significant activation by CDCA remained for both mutants. As expected, the double mutant almost completely lost its ability to respond to CDCA. The results indicated that different from human BSEP, FXR\textsubscript{1} regulated rat bsep through both IR1a and IR1b. Conversely, mutation in IR1a almost totally abolished FXR\textsubscript{2}-mediated transactivation as did the double mutant, indicating that FXR\textsubscript{2} regulates rat bsep through IR1a. Although mutation in IR1b decreased FXR\textsubscript{2}-mediated transactivation, significant induction by CDCA was detected with a comparable fold induction to the wt (7.7 versus 6.5) (Fig. 7D), indicating that similar to human BSEP, IR1b is required for basal activity but not for FXR\textsubscript{2}-mediated transactivation. Mutation in the half ER2 site had no detectable effect on FXR\textsubscript{1}- and FXR\textsubscript{2}-mediated transactivation (Fig. 7C, D), suggesting that different from human BSEP, the half ER2 in rat bsep does not play any roles in mediating FXR\textsubscript{2} transactivation.
Fig. 7. Mutational analysis of IR1a and IR1b in BSEP promoters. Four human BSEP promoter mutants with mutation in IR1a, IR1b, the half ER2 site, or both IR1a and IR1b were made using human BSEP promoter reporter phBSEP(-2.6kb) as parental template, resulting in mutants phBSEP(-2.6kb)-IR1a-Mut, phBSEP(-2.6kb)-IR1b-Mut, phBSEP(-2.6kb)-IR1ab-Mut, and phBSEP(-2.6kb)-ER2-Mut. Four equivalent mutants were constructed using rat bsep promoter reporter prBSEP(-2kb) as parental template, resulting in prBSEP(-2kb)-IR1a-Mut, prBSEP(-2kb)-IR1b-Mut, prBSEP(-2kb)-IR1ab-Mut, and prBSEP(-2kb)-ER2-Mut. The four human BSEP promoter mutants and wt were cotransfected with (A) FXRα1 or (B) FXRα2 into Huh 7 cells. The four rat bsep promoter mutants and wt were cotransfected with (C) FXRα1 or (D) FXRα2. Sixteen hours posttransfection, cells were treated with DMSO (0.1%) or CDCA (10 μM) for 30 h, followed by detection of luciferase activities by the dual-luciferase reporter assay system. The data are presented as mean ± SD of at least three repeated experiments. *P < 0.05 between DMSO and CDCA-treated cells (Student t-test). The fold inductions by CDCA over DMSO are presented on the top of the bars.
To determine whether FXR isoforms differentially bind to IR1a and IR1b in vitro, a series of EMSA assays were performed. With hIR1a and m/rIR1a probes, two distinct bands were shifted for both FXR/H92511 and FXR/H92512, indicating that two DNA/protein complexes were formed (Fig. 8A, B). To determine which band represents the specific binding of FXR/H92511 or FXR/H92512 to the probe, competition assays were carried out. The dominant bottom band was completely competed out by 10× unlabeled wt but not by mutated hIR1a or m/rIR1a oligonucleotide, indicating that the bottom band represented the probe-specific complex. To confirm that FXRα1 or FXRα2 is part of the complex, supershift assays with Abs against FXR were performed. Preincubation of FXR Abs with nuclear extracts markedly decreased the shifted band, suggesting that the binding of FXR Abs to FXR interferes with its binding to the hIR1a or m/rIR1a probe, decreasing the formation of the DNA/FXR complexes. The data thus demonstrated that both FXRα1 and FXRα2 bound to hIR1a or m/rIR1a abundantly in vitro. The abundant binding of FXRα1 to hIR1a was not expected since FXRα1 failed to transactivate hIR1a reporter.

With IR1b as a probe, two similar bands were shifted with FXRα1 and FXRα2. Competition EMSA assays concluded that the bottom band was the specific one. Incubation of FXR Abs with the complex resulted in a supershifted band (Fig. 8C), indicating that FXR is part of the complex. It should be emphasized that although similarly shifted patterns were observed for both FXRα1 and FXRα2, the bands shifted or supershifted with FXRα1 were much more abundant than those with FXRα2, suggesting that FXRα1 has a higher binding affinity to IR1b than does FXRα2.

FXRα1 and FXRα2 were specifically recruited to IR1b and IR1a, respectively, in human BSEP promoter in intact cells

Since IR1a and IR1b are located so close to each other, it was challenging to distinguish FXR recruitment to each individual site on the endogenous BSEP promoter. We used human BSEP promoter reporter wt and mutants to perform ChIP assays. Chromatins were prepared from Huh 7 cells cotransfected with FXRα1 or FXRα2 with human BSEP promoter wt, IR1a, IR1b, or IR1ab mutant. With FXRα1, a PCR band was readily detected in cells transfected...
with wt promoter when the chromatin was immunoprecipitated with anti-FXR Abs but not with IgG (Fig. 9A). More importantly, a PCR band with a relatively weaker signal was also detected in cells transfected with IR1a mutant. In contrast, no obvious bands were detected in cells transfected with either IR1b mutant or IR1ab double mutant (Fig. 9A). The data thus established that FXRα1 was specifically recruited to IR1b but not IR1a in intact Huh 7 cells. On the other hand, with FXRα2, a PCR band was readily detected in cells transfected with wt and IR1b mutant, whereas no signals were detected in cells transfected with IR1a and IR1ab double mutant (Fig. 9B). The data thus demonstrated that FXRα2 was specifically recruited to IR1a but not IR1b.

**FXRα1 and FXRα2 acted independently in transactivating human BSEP**

To investigate whether FXRα1 and FXRα2 have additive or competitive effects on the transactivation of human BSEP IR1a element reporter and full-length promoter, increasing amounts of FXRα1 were cotransfected with a constant amount of FXRα2, followed by detection of the transactivation levels. As shown in Fig. 10A, FXRα1 competitively decreased FXRα2-mediated transactivation in cells transfected with IR1a element reporter pGL3/p-2xhIR1a. In contrast, FXRα1 had minimal effects on FXRα2-mediated transactivation of the full-length promoter reporter phBSEP (-2.6kb), even at the highest dose (Fig. 10B). The results indicated that FXRα1 competitively bound to IR1a with FXRα2 in the IR1a element reporter, which is consistent with the data from EMSA (Fig. 8A). The results also indicated that no competitive binding of FXRα1 to IR1a occurred in the full-length promoter, which is consistent with the data from the ChIP assays (Fig. 9A). Such discrepancy in FXRα1’s effects on FXRα2-mediated transactivation of IR1a element and full-length promoter indicated that the context of IR1a played a determinant role in responding to FXRα1. It was thus concluded that in the context of full-length promoter, FXRα1 and FXRα2 transactivated human BSEP independently through IR1a and IR1b, respectively and that BSEP expression levels were predominately determined by FXRα2.

Combining the data from transactivation, mutational analysis, EMSA and ChIP assays, it was concluded that FXRα1 and FXRα2 regulated human BSEP independently through IR1b and IR1a. On the other hand, mouse and rat bsep are regulated by FXRα1 through both IR1a and IR1b and by FXRα2 through IR1a (Fig. 10C).

**Sequence differences between hIR1a and m/rIR1a were primarily responsible for the species-specific transactivation of BSEP by FXRα1 and FXRα2**

Sequence alignment of IR1a and IR1b in BSEP promoters from seven species including human, pongo pygmaeus, pan troglodytes, horse, dog, rat, and mouse revealed that IR1b was completely conserved (Fig. 11A), indicating the with wt promoter when the chromatin was immunoprecipitated with anti-FXR Abs but not with IgG (Fig. 9A). More importantly, a PCR band with a relatively weaker signal was also detected in cells transfected with IR1a mutant. In contrast, no obvious bands were detected in cells transfected with either IR1b mutant or IR1ab double mutant (Fig. 9A). The data thus established that FXRα1 was specifically recruited to IR1b but not IR1a in intact Huh 7 cells. On the other hand, with FXRα2, a PCR band was readily detected in cells transfected with wt and IR1b mutant, whereas no signals were detected in cells transfected with IR1a and IR1ab double mutant (Fig. 9B). The data thus demonstrated that FXRα2 was specifically recruited to IR1a but not IR1b.

**FXRα1 and FXRα2 acted independently in transactivating human BSEP**

To investigate whether FXRα1 and FXRα2 have additive or competitive effects on the transactivation of human BSEP IR1a element reporter and full-length promoter, increasing amounts of FXRα1 were cotransfected with a constant amount of FXRα2, followed by detection of the transactivation levels. As shown in Fig. 10A, FXRα1 competitively decreased FXRα2-mediated transactivation in cells transfected with IR1a element reporter pGL3/p-2xhIR1a. In contrast, FXRα1 had minimal effects on FXRα2-mediated transactivation of the full-length promoter reporter phBSEP (-2.6kb), even at the highest dose (Fig. 10B). The results indicated that FXRα1 competitively bound to IR1a with FXRα2 in the IR1a element reporter, which is consistent with the data from EMSA (Fig. 8A). The results also indicated that no competitive binding of FXRα1 to IR1a occurred in the full-length promoter, which is consistent with the data from the ChIP assays (Fig. 9A). Such discrepancy in FXRα1’s effects on FXRα2-mediated transactivation of IR1a element and full-length promoter indicated that the context of IR1a played a determinant role in responding to FXRα1. It was thus concluded that in the context of full-length promoter, FXRα1 and FXRα2 transactivated human BSEP independently through IR1a and IR1b, respectively and that BSEP expression levels were predominately determined by FXRα2.

Combining the data from transactivation, mutational analysis, EMSA and ChIP assays, it was concluded that FXRα1 and FXRα2 regulated human BSEP independently through IR1b and IR1a. On the other hand, mouse and rat bsep are regulated by FXRα1 through both IR1a and IR1b and by FXRα2 through IR1a (Fig. 10C).

**Sequence differences between hIR1a and m/rIR1a were primarily responsible for the species-specific transactivation of BSEP by FXRα1 and FXRα2**

Sequence alignment of IR1a and IR1b in BSEP promoters from seven species including human, pongo pygmaeus, pan troglodytes, horse, dog, rat, and mouse revealed that IR1b was completely conserved (Fig. 11A), indicating the with wt promoter when the chromatin was immunoprecipitated with anti-FXR Abs but not with IgG (Fig. 9A). More importantly, a PCR band with a relatively weaker signal was also detected in cells transfected with IR1a mutant. In contrast, no obvious bands were detected in cells transfected with either IR1b mutant or IR1ab double mutant (Fig. 9A). The data thus established that FXRα1 was specifically recruited to IR1b but not IR1a in intact Huh 7 cells. On the other hand, with FXRα2, a PCR band was readily detected in cells transfected with wt and IR1b mutant, whereas no signals were detected in cells transfected with IR1a and IR1ab double mutant (Fig. 9B). The data thus demonstrated that FXRα2 was specifically recruited to IR1a but not IR1b.

**FXRα1 and FXRα2 acted independently in transactivating human BSEP**

To investigate whether FXRα1 and FXRα2 have additive or competitive effects on the transactivation of human BSEP IR1a element reporter and full-length promoter, increasing amounts of FXRα1 were cotransfected with a constant amount of FXRα2, followed by detection of the transactivation levels. As shown in Fig. 10A, FXRα1 competitively decreased FXRα2-mediated transactivation in cells transfected with IR1a element reporter pGL3/p-2xhIR1a. In contrast, FXRα1 had minimal effects on FXRα2-mediated transactivation of the full-length promoter reporter phBSEP (-2.6kb), even at the highest dose (Fig. 10B). The results indicated that FXRα1 competitively bound to IR1a with FXRα2 in the IR1a element reporter, which is consistent with the data from EMSA (Fig. 8A). The results also indicated that no competitive binding of FXRα1 to IR1a occurred in the full-length promoter, which is consistent with the data from the ChIP assays (Fig. 9A). Such discrepancy in FXRα1’s effects on FXRα2-mediated transactivation of IR1a element and full-length promoter indicated that the context of IR1a played a determinant role in responding to FXRα1. It was thus concluded that in the context of full-length promoter, FXRα1 and FXRα2 transactivated human BSEP independently through IR1a and IR1b, respectively and that BSEP expression levels were predominately determined by FXRα2.

Combining the data from transactivation, mutational analysis, EMSA and ChIP assays, it was concluded that FXRα1 and FXRα2 regulated human BSEP independently through IR1b and IR1a. On the other hand, mouse and rat bsep are regulated by FXRα1 through both IR1a and IR1b and by FXRα2 through IR1a (Fig. 10C).

**Sequence differences between hIR1a and m/rIR1a were primarily responsible for the species-specific transactivation of BSEP by FXRα1 and FXRα2**

Sequence alignment of IR1a and IR1b in BSEP promoters from seven species including human, pongo pygmaeus, pan troglodytes, horse, dog, rat, and mouse revealed that IR1b was completely conserved (Fig. 11A), indicating the
...through m/rIR1a. In mouse and rat, FXR regulates BSEP exclusively through IR1b and hIR1a, respectively. In human, FXR regulates BSEP through IR1a and IR1b. In human, FXR regulates BSEP through IR1a and IR1b. In human, FXR regulates BSEP through IR1a and IR1b.

A

B

C

Fig. 10. Interrelationship between FXRα1 and FXRα2. The effects of FXRα1 on FXRα2-mediated transactivation of (A) human IR1a element reporter pGL3/p-2xhIR1a and (B) full-length human BSEP promoter reporter phBSEP(-2.6kb) were investigated. Increasing amounts of FXRα1 were cotransfected with a constant amount of FXRα2 and pGL3/p-2xhIR1a or phBSEP(-2.6kb). Vector pcDNA5 plasmid DNAs were also cotransfected to obtain a constant total plasmid DNA in each well. Sixteen hours posttransfection, cells were treated with DMSO (0.1%) or CDCA (10 μM) for 30 h, followed by detection of luciferase activity. The data are presented as mean ± SD of at least three repeated experiments. (C) A diagram summarizing FXRα1- and FXRα2-mediated transactivation of BSEP through IR1a and IR1b. In human, FXRα1 and FXRα2 regulate BSEP exclusively through IR1b and hIR1a, respectively. In mouse and rat, FXRα1 is able to transactivate BSEP through both m/rIR1a and IR1b, whereas FXRα2 transactivates exclusively through m/rIR1a.

DISCUSSION

As the limiting step in bile acid enterohepatic circulation, canicular efflux of bile acids by BSEP is tightly regulated by the bile acids/FXR signaling pathway. In our previous study, it was found that human BSEP was isoform dependently regulated by FXR, with FXRα2 being the predominant regulator. The relative expression of FXRα1 and FXRα2 was altered in HCC patients and associated with dysregulation of BSEP (25). Similar FXR isoform-dependent transactivation has been reported for other FXR targets, including ileal bile acid-binding protein (24), fibrinogens (26), syndecan-1 (27), and α-crystallin (31). However, it is currently not understood how FXRα1 and FXRα2 exhibit different activity on those FXR targets. Based on EMSA data, it was postulated that FXRα2 had a higher binding affinity than FXRα1 to the FXRE of the targets (24). Such notion is consistent with the fact that...
Fig. 11. IR1b is completely conserved among species, whereas IR1a is primarily responsible for the species difference in transactivation of BSEP by FXRα1 and FXRα2. (A) Sequences of the IR1a and IR1b from seven species including mouse, rat, dog, cat, horse, primate, and human were aligned. The overall conservation percentages of IR1a and IR1b are indicated. (B) The hIR1a in human BSEP promoter reporter phBSEP(-2.6kb) was replaced with mouse/rat m/rIR1a, generating a chimeric reporter pBSEP(-2.6kb)-m/rIR1a. (C) The rIR1a in rat bsep promoter reporter prBSEP(-2kb) was replaced with human hIR1a, resulting a chimeric reporter prBSEP(-2kb)-hIR1a. Human and rat bsep promoter wt and chimeric reporters were cotransfected with FXRα1 or FXRα2 into Huh 7 cells, followed by treatment of transfected cells with DMSO (0.1%) or CDCA (10 μM) for 30 h. Reporter activities were detected by the dual-luciferase reporter assay system. The data are presented as mean ± SD of at least three repeated experiments. (D) Female CD-1 mice were randomly divided into four groups. Each group of mice was hydrodynamically injected with either phBSEP(-2.6kb),
the four amino acid residues inserted in FXRα1 are located immediately after the DNA binding domain (DBD). The insertion may alter the conformational structure in DBD, thus compromising its ability to bind to FXRE. However, in this study, we showed that FXRα1 and FXRα2 transactivated human BSEP through two FXREs, IR1b and IR1a, respectively. The question remains how FXRα1-mediated transactivation through IR1b is much weaker than FXRα2-mediated activation through IR1α in human BSEP. One possible explanation is that FXRα1 might have a weaker intrinsic activity than FXRα2. However, such notion is inconsistent with the results that FXRα1 transactivated mouse and rat bsep as potently as did FXRα2 (Fig. 1). Another more likely explanation is that the sequence and/or the content of the IR1b are not optimal for maximal activation by FXRα1.

Considering that FXRα2-mediated transactivation through IR1α is the predominant pathway regulating human BSEP expression, the question remains regarding the physiological significance of FXRα1-mediated transactivation through IR1b in regulating human BSEP. One possible physiological function of FXRα1/IR1b pathway is to support the maximal transactivation of BSEP by the FXRα2/IR1α pathway. Consistent with such speculation are results from the mutational studies that mutation in IR1b markedly reduced FXRα2-mediated basal as well as CDDA-induced transactivation of human BSEP (Fig. 7B). Searching the IR1b sequence in the database revealed that IR1b element is present in many promoter regions (data not shown). However, it remains to be determined whether IR1b element is involved in regulating any of those genes.

Our results showed that in addition to total FXR levels, relative FXR isoform expression levels (FXRα1/FXRα2 ratios) significantly affected human BSEP expression. In healthy human liver tissues, FXRα1/FXRα2 ratios ranged from 1.09 to 1.63, with an average of 1.37, while BSEP expression exhibited 24-fold differences. It was our expectation that as the FXRα1/FXRα2 ratios increased, BSEP expression would decrease. However, the actual data revealed that the relationship between FXRα1/FXRα2 ratio and BSEP expression was dependent on the total FXR expression levels (Fig. 2A, B). As expected, human BSEP expression decreased as the ratios increased at the higher levels of total FXR. However, in contrast to our expectation, BSEP expression increased as the FXRα1/FXRα2 ratio increased at the lower levels of total FXR. We were not able to provide a satisfactory explanation for the relationship exhibited at the lower levels of total FXR. Such phenomenon may be related to FXRα1’s specific activation through IR1b. Since IR1b is completely conserved among species, it is reasonable to speculate that FXRα1-mediated transactivation of human BSEP through IR1b is critical for maintaining BSEP expression in the events of low total FXR levels.

Although FXRα1 abundantly bound to hIR1a and FXRα2 weakly associated with IR1b in vitro in EMSAs (Fig. 8A, C), and FXRα1 competitively decreased FXRα2-mediated transactivation of IR1α reporter (Fig. 10A), it was concluded that FXRα1 and FXRα2 did not bind to hIR1a and IR1b in the full-length promoter content in intact cells, respectively, based on the following considerations: first, recruitments of FXRα1 to hIR1a and FXRα2 to IR1b were not detected in intact cells with the ChIP assays (Figs. 9A, B); second, FXRα1 failed to transactivate hIR1a reporter (Fig. 5A); and third, overexpression of FXRα1 had a negligible effect on FXRα2-mediated transactivation of the full-length promoter (Fig. 10B), indicating no competition between the two for binding to IR1a. Taken together, the data strongly supported the conclusion that FXRα1 and FXRα2 exclusively bind to IR1b and IR1a, respectively, in intact cells. Such discrepancy of FXR binding to FXRE between in vitro and in intact cells indicates that FXR binding to FXRE within cells is governed not only by the sequence but also by the content. Such notion is supported by the finding that among the 1.7 million FXREs predicted in the mouse genome, only 1,656 (32) or 7,794 (30) FXREs were detected for FXR binding in vivo in the liver.

In the ChIP assays, it was noticed that compared with wt promoter, the recruitment of FXRα1 to IR1b in the IR1α mutant or FXRα2 to IR1α in the IR1b mutant was decreased (Fig. 9A, B). One possible explanation for such phenomenon is that mutation in one site has a negative impact on the binding of FXRα1 or FXRα2 to the other site due to the proximity of the two sites (18 bases apart). It should be noted that the ChIP assays were carried out on the transfected wt and mutant promoters. Definitive evidence for FXRα1 binding to IR1b or FXRα2 binding to IR1a in the endogenous BSEP promoter remains to be provided.

In contrast to human BSEP, mouse and rat bsep were comparably transactivated by FXRα1 and FXRα2 (Fig. 1). Mouse and rat m/rIR1a is able to support both FXRα1 and FXRα2-mediated transactivation as the FXRE consensus (FXREc: AGGTCA CTGAGCT) does (data not shown). Comparing the sequences of hIR1a, IR1b, and m/rIR1a with FXREc, m/rIR1a is the closest to FXREc, followed by IR1b and hIR1a. Detailed analysis of the sequences revealed that a nucleotide G at position 2 is conserved among FXREc, m/rIR1a, and hIR1a but altered to T in IR1b. Therefore, the G at position 2 may play a determinant role in mediating FXRα2 transactivation. On the other hand, nucleotides A and T in positions 1 and 13 are conserved.
among FXREs, m/rIR1a, and IR1b, but they are substituted with G and C in hIR1a, suggesting that the A and T in positions 1 and 13 are important for mediating FXRα transactivation. In this study, we also identified a half ER2 site immediately upstream of IR1a (Fig. 5C). Characterization of the ER2 half site revealed that it played an important role in achieving maximal transactivation of human BSEP by FXR, but the site had no effects on FXRβ-mediated transactivation of rat bsep. Such difference in requirement of ER2 for FXRα to achieve maximal BSEP transactivation may relate to the fact that human hIR1a is more remote than mouse/rat m/rIR1a to the FXRE consensus. The imperfect hIR1a needs the half ER2 site to support maximal FXRα transactivation, whereas the optimal m/rIR1a alone has the capability of fully supporting FXRα transactivation.

In addition to the important role played by FXR in regulating bile acid homeostasis (33, 34), FXR regulates a myriad of other target genes critical for cholesterol, lipid, and glucose homeostasis (34–37), liver regeneration (38), and tumorigenesis (39, 40). Based on our new finding that FXRα1 and FXRα2 transactivated human BSEP through two distinct FXREs, it is reasonable to speculate that FXRα1 and FXRα2 may preferably regulate different sets of FXR target genes involved in various functional pathways. We are currently searching for those FXR isoform-specific target genes.

The authors thank Drs. David Mangelsdorf and Jonathan Reicher for providing human FXRα expression plasmid and rat HCC L0 cells, respectively. Technical and instrumental support from the RI-INBRE Core Facility are greatly appreciated.

REFERENCES


