The nuclear receptor for bile acids, FXR, transactivates human organic solute transporter-α and -β genes

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Submitted 14 September 2005; accepted in final form 31 October 2005

Landrier, Jean-François, Jyrki J. Eloranta, Stephan R. Vavricka, and Gerd A. Kullak-Ublick. The nuclear receptor for bile acids, FXR, transactivates human organic solute transporter-α and -β genes. Am J Physiol Gastrointest Liver Physiol 290: G476–G485, 2006. First published November 3, 2005; doi:10.1152/ajpgi.00430.2005.—Bile acids are synthesized from cholesterol in the liver and excreted into bile via the hepatocyte canalicular bile salt export pump. After their passage into the intestine, bile acids are reabsorbed in the ileum by sodium-dependent uptake across the apical membrane of enterocytes. At the basolateral domain of ileal enterocytes, bile acids are extruded into portal blood by the heterodimeric organic solute transporter OSTα/OSTβ. Although the transport function of OSTα/OSTβ has been characterized, little is known about the regulation of its expression. We show here that human OSTα/OSTβ expression is induced by bile acids through ligand-dependent transactivation of both OST genes by the nuclear bile acid receptor/farnesoid X receptor (FXR). FXR agonists induced endogenous mRNA levels of OSTα and OSTβ in cultured cells, an effect that was not discernible upon inhibition of FXR expression by small interfering RNAs. Furthermore, OST mRNAs were induced in human ileal biopsies exposed to the bile acid chenodeoxycholic acid. Reporter constructs containing OSTα or OSTβ promoters were transactivated by FXR in the presence of its ligand. Two functional FXR binding motifs were identified in the OSTα gene and one in the OSTβ gene. Targeted mutation of these elements led to reduced inducibility of both OST promoters by FXR. In conclusion, the genes encoding the human OSTα/OSTβ complex are induced by bile acids and FXR. By coordinated control of OSTα/OSTβ expression, bile acids may adjust the rate of their own efflux from enterocytes in response to changes in intracellular bile acid levels.

bile acid homeostasis; enterohepatic circulation; nuclear receptors

THE ENTEROHEPATIC CIRCULATION of bile acids depends on coordinated interplay between specialized transport proteins that are located at the apical and basolateral domains of intestinal epithelial cells (enterocytes) and hepatocytes (12). In hepatocytes, bile acids are synthesized from cholesterol and are excreted as monovalent anions into bile via the bile salt export pump (BSEP) located at the canalicular hepatocyte membrane. Divalent sulfated or glucuronidated bile acids are excreted into bile via canalicular multidrug resistance protein 2 (MRP2). After their passage into the intestine, bile acids are reabsorbed with high efficiency in the ileum. The key uptake system from the intestinal lumen into enterocytes is the apical sodium-dependent bile acid transporter (ASBT) (25, 31). Human ASBT transports both conjugated and unconjugated bile acids (3). After their uptake into enterocytes, bile acids may bind to, and be intracellularly transported by, ileal bile acid-binding protein (I-BABP) (7, 14).

The mechanism by which bile acids are transported across the basolateral membrane of enterocytes into portal blood has been recently elucidated (4). It was shown that organic solute transporter (OST)α and OSTβ form a heterodimer and mediate the apical efflux of taurocholate in transfected canine kidney cells. In the mouse intestine, both OSTα and OSTβ mRNAs are most abundant in the ileum, the main site of intestinal bile acid reabsorption. By immunofluorescence microscopy, Ostα and Ostβ proteins were localized to lateral and basolateral membranes of ileocytes, with a similar vertical gradient of expression along the intestinal crypt-to-villus axis to what has been shown for mouse Asbt.

Ostα and Ostβ were originally isolated from a skate liver cDNA library by functional screening based on [3H]taurocholate uptake in Xenopus laevis oocytes injected with cRNA pools (29). Ostα/Ostβ-mediated transport was Na⁺ independent, saturable, and inhibited by organic anions and steroids. Subsequently, human and mouse orthologs were identified and shown to possess a similar substrate profile to skate Ostα/Ostβ (24). Human OSTα and OSTβ mRNAs are most abundant in the small intestine, kidney, and liver, the same tissues that express ASBT. The amino acid identity between human OSTα and mouse Ostα is 83%, and there is a 41% amino acid identity between skate Ostα and mammalian orthologs. For Ostβ, the degree of identity is 62.5% between humans and mice and 25–29% between skate Ostβ and mammalian orthologs. The predicted amino acid sequences of OST proteins are not homologous to those of any previously identified members of the solute carrier gene family (24, 29). It is unclear how OSTα and OSTβ interact at the cell membrane to generate an active transporter complex. However, both are integral membrane proteins with predicted similar topology to sensory rhodopsins, namely, a seven-helix transmembrane protein (OSTα) and an accessory protein (OSTβ) with one to two transmembrane domains (24, 29).

Although the transport function, tissue distribution, and subcellular location of the OSTα/OSTβ complex have been characterized, little is known about the regulation of its expression. In view of its function as a bile acid export system at the basolateral membrane of enterocytes, we hypothesized that bile acids may transcriptionally regulate the expression of OSTα/OSTβ genes, as previously shown for several other bile acid transporters, notably BSEP (1, 20, 22). This hypothesis was strengthened by the finding that Ostα/Ostβ mRNA levels are decreased in the ileum but increased in the cecum and proximal

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colon of mice that lack expression of the ileal bile acid transporter Asbt (4). Ileocytes of Asbt-null mice have decreased uptake of bile acids, leading to increased influx of bacterially deconjugated bile acids into enterocytes. Bile acids are ligands of the nuclear bile acid receptor/farnesoid X receptor (FXR) (16, 19, 28), a member of the nuclear receptor family of transcription factors. Ligand-activated FXR induces most target promoters through direct binding to inverted hexameric nucleotide repeat (IR) separated by one nucleotide (IR-1) motifs (13). In most cases, FXR binds DNA as a dimer with retinoid X receptor (RXR)-α, a nuclear receptor known to productively heterodimerize with a range of other members of the nuclear receptor family (17). We show here that human OSTα and OSTβ genes are direct targets of the FXR-RXRα heterodimer, resulting in increased gene expression in the presence of bile acids. Our data complete the loop of FXR-regulated bile acid transporter genes within the enterohepatic circulation and further emphasize the role of FXR as a master regulator of bile acid transport at all plasma membrane domains in enterocytes and hepatocytes (6, 27).

**MATERIALS AND METHODS**

**Chemicals.** GW4064 was a gift from Dr. Daniel Berger (GlaxoSmithKline). Deoxyadenosine 5'-[α-32P]triphosphate ([α-32P]dATP; 6,000 Ci/mmol) was purchased from Amersham Biosciences Europe (Otellingen, Switzerland). Restriction enzymes were from Roche Diagnostics (Rotkreuz, Switzerland), Pfu Turbo DNA polymerase was from Stratagene (La Jolla, CA), and the LigAFast Rapid Ligation Kit was from Promega Catalys (Wallisellen, Switzerland). Oligonucleotides were synthesized by Microsynth (Balgach, Switzerland). Other chemicals were purchased from Sigma (Buchs, Switzerland) unless stated otherwise.

**Cell culture.** Human hepatoma cell lines Huh7 and HepG2 (American Type Culture Collection; Molsheim, France) were cultured in RPMI 1640 medium (Sigma) supplemented with 10% FBS (Sigma) and 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen; Basel, Switzerland). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO2.

**RNA isolation, reverse transcription, and real-time PCR.** Hub7 or HepG2 cells were seeded in six-well plates and, when 80% confluent, treated with 50 μM chenodeoxycholic acid (CDCA), 200 nM of the FXR agonist GW4064, or the vehicle DMSO. Each PCR was performed in triplicate, and all experiments were repeated three times.

**Table 1. Sequences of oligonucleotides used for cloning, mutagenesis, and real-time PCR and as EMSA probes and competitors**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5'-3')</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>hOSTα -1475 for</td>
<td>GACTGAGCTTCTGAACTTCTAAGAACATCCCCAG (SacI)</td>
<td>hOSTα (-1475/+161) cloning</td>
</tr>
<tr>
<td>hOSTα +161 rev</td>
<td>GACTGAGCTCAGCAATTTTCAGACAGCGGTCATGCTTC (NheI)</td>
<td>hOSTα (-1475/+161) cloning</td>
</tr>
<tr>
<td>hOSTβ -4748 for</td>
<td>GATGGTAGCCTTCTATCTGACCTGAGTCATGCTTC (KpnI)</td>
<td>hOSTβ (-4748/+29) cloning</td>
</tr>
<tr>
<td>hOSTβ +29 rev</td>
<td>GACTGAGCTCAGCAATTTTCAGACAGCGGTCATGCTTC (NheI)</td>
<td>hOSTβ (-4748/+29) cloning</td>
</tr>
<tr>
<td>hOSTα mut1</td>
<td>CAGTGGGCTGGGCCTGAATGAGGTGGCGAACGTGCC</td>
<td>hOSTα (-1475/+161) mut1 mutagenesis</td>
</tr>
<tr>
<td>hOSTα mut2</td>
<td>GGGTGGGCCGCTGGGAGTGAGGGGCGGAGGGGAGG</td>
<td>hOSTα (-1475/+161) mut2 mutagenesis</td>
</tr>
<tr>
<td>hOSTβ mut</td>
<td>CAGTGGGCTGGGCCTGAATGAGGTGGCGAACGTGCC</td>
<td>hOSTβ (-4748/+29) mut mutagenesis</td>
</tr>
</tbody>
</table>

*Only the top strands are shown for oligonucleotides used in EMSA assays. Where applicable, the restriction sites introduced are underlined, and the corresponding enzymes used are given in parentheses. hOST, human organic solute transporter; for, forward; rev, reverse; mut, mutant; wt, wild type; I-BABP, ileal bile acid binding protein; FXR, farnesoid X receptor.*
of TransIT-TKO transfection reagent (Dharmacon; Lafayette, CO) were mixed with 250 μl of serum-free OptiMEM (Invitrogen) followed by the addition of either the SMARTpool short interfering RNA (siRNA) targeting FXR (Dharmacon) or siCONTROL nontargeting siRNA no. 1 (Dharmacon) to a final concentration of 50 nM. After an incubation at room temperature for 20 min, siRNA mixtures were added to 1,250 μl RPMI 1640 medium supplemented with 10% FBS in each well. Twelve hours after transfection, cells were treated for further 24 h with 50 μM CDCA or 200 nM GW4064. Cellular RNAs were extracted using TRIzol reagent and subjected to real-time PCR as described above.

Preparation of protein extracts and immunoblot analysis. To prepare whole cell extracts, cells on six-well plates were washed with ice-cold PBS and lysed by a 5-min incubation in 250 μl of ice-cold lysis buffer (containing 50 mM Tris·HCl (pH 8.0), 150 mM NaCl, 1% (vol/vol) Igepal CA-630, 0.5% (wt/vol) Na-deoxycholate, 1 mM EDTA, 0.1% (vol/wt) SDS, and 1% (vol/vol) glycerol) supplemented with Complete protease inhibitors (Roche Diagnostics). Debris was removed by centrifugation at 14,000 rpm for 30 min at 4°C. Protein concentrations were determined with the bicinchoninic acid protein assay (Pierce), and samples were stored at −80°C until usage. Three micrograms of protein extracts were separated on 10% SDS polyacrylamide gels and electrophoretically transferred onto nitrocellulose membranes (Hybond ECL; Amersham Biosciences Europe). Membranes were blocked overnight in 5% (wt/vol) nonfat milk in PBS-Tween 20 [0.1% (vol/vol) Tween 20 in PBS; PBS-T]. After this, membranes were probed with an antibody against FXR (H-130, Santa Cruz Biotechnology; Nunningen, Switzerland) at a concentration of 0.4 μg/ml in 5% (wt/vol) nonfat milk-PBS-T for an hour. After membranes were washed three times with 5% (wt/vol) nonfat milk-PBS-T, horseradish peroxidase-conjugated goat anti-rabbit antibody (Pierce) was added at a concentration of 10 ng/ml in 5% (wt/vol) nonfat milk-PBS-T for 1 h. Blots were then washed three times with 5% (wt/vol) nonfat milk-PBS-T and twice with PBS, followed by detection with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) and exposure on Hyperfilm ECL (Amersham Biosciences Europe). To verify equal loading of the protein samples, blots were stripped with Restore Western Blot Stripping Solution (Pierce), reblocked, and reprobed for constitutively expressed Ku-70 antigen. Ku-70 probing and detection were performed as described above except that the Ku-70 antibody (C-19, Santa Cruz Biotechnology) was used at a concentration of 50 ng/ml and horseradish peroxidase-conjugated rabbit anti-goi antibody (DakoCytomation; Zug, Switzerland) was used as the secondary antibody at a concentration of 167 ng/ml.

EMSAs. Oligonucleotides used in EMSAs were designed to have a 5′-TCGA overhang at both ends when annealed, allowing radioactive labeling by fill-in reactions. Fifty nanograms of annealed oligonucleotides were labeled in 20-μl reactions containing 200 units SuperScript II RNase H− Reverse Transcriptase (Invitrogen), 1× First-Strand Buffer (Invitrogen), 10 mM DTT, 250 mM dGTP/dCTP/dTTP, and 20 μCi [α-32P]dATP (Amersham Biosciences). Unincorporated nucleotides were removed using Microspin G-25 columns (Amersham Biosciences). Two microliters (Fig. 6, A–C) or 0.5 μl (Fig. 6, D–F) of the FXR and RXRα proteins synthesized using the TNT rabbit reticulocyte lysate-coupled in vitro transcription/translation system (Promega Catalys) or 5 μg of Huh7 nuclear extracts (Fig. 6, D–F) prepared using the NE-PER extraction kit (Pierce) were used for DNA binding reactions. Approximately 30,000 counts/min (0.5–10 ng) of the radioactive probes were used per reaction. Protein-DNA complexes were formed in binding buffer (10 mM Tris·HCl (pH 8.0), 40 mM KCl, 0.05% (vol/vol) Igepal CA-630, 6% (vol/vol) glycerol, 1 mM DTT, and 50 mM poly(dl-dC)-poly(dl-dC)) in a total volume of 20 μl for 30 min at 4°C. In supershift experiments, 1 μg of FXR (C-20, Santa Cruz Biotechnology) and/or RXRα (ΔN197, Santa Cruz Biotechnology) antibodies were added to the extracts 30 min before the probe and incubated at 4°C. In competition experiments, a 50-fold molar excess of the competing oligonucleotides was added simultane-

ously with the radioactive probe. Immediately after the binding reactions, samples were loaded onto preelectrophoresed 5% (acryl-
amide/bis 30:1) native acrylamide gels and run at 200 V in 0.5× 44 mM Tris·borate, 1 mM EDTA (TBE) for 1.5 h. Gels were then fixed in 10% (vol/vol) acetic acid for 10 min, rinsed with water, dried onto Whatman DE81 paper under vacuum, and exposed to Kodak BioMax MR-1 films (Sigma) at −70°C.

REPORTER GENE ASSAYS. On the basis of sequences available in the National Center for Biotechnology Information (NCBI) database, fragments corresponding to the putative promoter regions of the human OSTα and OSTβ genes were PCR amplified using the primers shown in Table 1. PCR products were cloned into the luciferase (Luc) vector pGL3basic (Promega Catalys) to generate OSTα (-1475/+161) and OSTβ (-4748/+29) reporter constructs. Point mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene) and the oligonucleotides listed in Table 1. The sequences of all constructs were confirmed by DNA sequencing (Microsynth). The mammalian expression plasmids pCMX-FXR and pCMX-RXRα were provided by Dr. D. Mangelsdorf (University of Texas Southwestern Medical Center, Dallas, TX).

Transfection efficiencies and reporter assays. Cells were seeded in 48-well plates at a density of 1×10⁵ cells/well and transfection with 400 ng of the indicated Luc reporter constructs and 100 ng of the indicated expression plasmids using FuGENE 6 reagent (Roche Diagnostics) at a ratio of 3 μl FuGENE 6 per 1 μg DNA. To normalize the amount of DNA transfected, pcDNA3.1 vector (Invitrogen) was added where appropriate. To control for transfection efficiency, 100 ng of a pSV-β-galactosidase reporter plasmid (Promega Catalys) were cotransfected. Twelve hours after transfection, the medium was supplemented with 200 nM GW4064 or the vehicle DMSO. Cells were harvested 36 h after transfection, and Luc activities determined using the Luciferase Assay System (Promega Catalys) in a Lumat LB 9507–2 luminometer (Berthold Technologies; Regensdorf, Switzerland). β-Galactosidase activities were quantified by a chlorophenol-
red-β-D-galactopyranoside-based colorimetric assay in a microplate reader (Molecular Devices, Bucher Biotech; Basel, Switzerland). Reporter activities obtained for empty pGL3basic corresponding to each test condition as well as for the test construct containing the test promoter in the control conditions were set to 1, and fold activities are shown relative to this. Transfection experiments were performed at least three times, and the results are shown as mean values ± SD.

RESULTS

FXR agonists induce human OSTα and OSTβ mRNA expression. To study the influence of bile acid treatment on endogenous expression of human OSTα and OSTβ mRNAs, hepatocyte-derived Huh7 and HepG2 cells were grown in the presence or absence of 50 μM CDCA for 24 h. These cell lines were chosen for the in vitro analysis because they exhibit detectable levels of OSTα and OSTβ mRNAs in standard cell culture conditions. CDCA treatment led to a strong induction of both OSTα and OSTβ mRNA levels as measured by quantitative PCR (Fig. 1A). Bile acids, such as CDCA, regulate gene transcription by serving as agonistic ligands for the bile acid receptor FXR (16, 19, 28). However, bile acids can also affect the expression of specific genes via FXR-independent pathways (26, 32). To investigate whether the induction of OSTα and OSTβ expression by CDCA involves a FXR-dependent mechanism, we treated Huh7 and HepG2 cells with the synthetic and specific FXR ligand GW4064 (30) in parallel with CDCA. Whereas GW4064 should enhance FXR-dependent gene transcription, it does not affect FXR-independent pathways. As shown in Fig. 1B, treatment of the cells with
GW4064 also increased mRNA levels of both OST\(\alpha\) and OST\(\beta\), supporting the role of FXR in inducing their expression.

The bile acid CDCA induces OST mRNA expression in human ileal tissue. Having shown that treatment of cultured cells with the bile acid CDCA increases the expression of both human OST mRNAs, we next investigated whether this induction can be reproduced in human tissue samples. Thus ileal biopsies were obtained from five healthy patients and treated with either CDCA or the vehicle DMSO for 4 h before the relative OST mRNA levels were determined. In ileal biopsies from all five patients, both OST\(\alpha\) and OST\(\beta\) mRNAs were consisently increased upon CDCA treatment (Fig. 2). The magnitude of induction of both OST genes by CDCA was less pronounced in biopsies than in cultured cell lines. We propose that this is attributable to the considerably shorter incubation time of biopsies (4 h) than of cultured cells (24 h) in CDCA-containing medium. Incubation periods exceeding 4 h led to gradual deterioration of ileal tissue, as judged by microscopic inspection and by the integrity of isolated RNA. As a positive control, using the same biopsy mRNAs, we observed similar CDCA-dependent fold increases in mRNA derived from the small heterodimer partner (SHP) gene (data not shown), a known target for transcriptional activation by FXR (8, 15).

**FXR-targeting siRNAs inhibit OST mRNA induction by bile acids and GW4064.** To confirm that FXR is essential for the induction of human OST\(\alpha\) and OST\(\beta\) genes by bile acids and GW4064, we next examined the effect of reducing endogenous FXR expression in Huh7 cells by transfecting a pool of four FXR-specific siRNAs. Nontargeting siRNAs designed not to affect the expression of any known human genes were used to control for nonspecific effects. As anticipated, the transfection of the FXR-specific siRNA pool led to an \(\sim 70\%\) reduction in the endogenous FXR mRNA expression level in cells treated with the vehicle DMSO, CDCA, or GW4064 (Fig. 3A). Similarly, the level of FXR protein was strongly reduced in cells transfected with FXR-specific siRNAs, whereas the expression of constitutive Ku-70 protein was unaffected (Fig. 3B). In support of the crucial role for FXR, the induction of both

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**Fig. 1.** Human organic solute transporter (hOST)\(\alpha\) and hOST\(\beta\) genes are induced by farnesoid X receptor (FXR) agonists. Human hepatoma Huh7 and HepG2 cells were treated with 50 \(\mu\)M chenodeoxycholic acid (CDCA; A), 200 nM GW4064 (B), or the vehicle DMSO for 24 h. Relative OST\(\alpha\) and OST\(\beta\) mRNA levels were quantified by real-time PCR. In each experiment, the level of OST mRNA expression obtained for DMSO-treated cells is set to 1, and OST mRNA expression in other test conditions is shown relative to this. The average threshold cycles of the DMSO control samples were 32.7 \(\pm\) 1.7 for OST\(\alpha\) and 29.5 \(\pm\) 1.2 for OST\(\beta\) in Huh7 cells and 27.4 \(\pm\) 0.2 for OST\(\alpha\) and 31.5 \(\pm\) 0.5 for OST\(\beta\) in HepG2 cells.
OSTα and OSTβ mRNA expression by CDCA and GW4064 was abolished in cells treated with FXR-specific siRNAs but was not affected by nontargeting siRNAs (Fig. 3, C and D). As a positive control, the induction of the known FXR target gene SHP (8, 15) by CDCA and GW4064 was efficiently blocked by FXR-specific siRNAs (data not shown). The mRNA expression of another transporter, MDR1, or of another member of the steroid/nuclear receptor family, the glucocorticoid receptor (GR), was not affected by treatment of Huh7 cells with FXR siRNAs (data not shown), indicating that these specifically target FXR.

Identification of FXR-responsive elements within OST promoters. To identify the promoter sequences of human OSTα and OSTβ genes, we performed a BLAST search against human genomic sequences using cDNA sequences for human OSTα and OSTβ genes in the NCBI database (NM_152672 and NM_178859, respectively). Bacterial artificial chromosome clones RP11-447L10 and RP11-325L2 were found to contain the 5'-untranslated regions and regions upstream of the predicted transcriptional initiation sites of OSTα (GenBank NT_029928) and OSTβ (Gen Bank NT_010194) genes, respectively. To search for FXR-responsive elements (FXREs) that may mediate induction by bile acids and GW4064, these putative regulatory regions were subjected to in silico analysis with two Web-based algorithms: NUBIScan (21) and MatInspector (http://www.genomatix.de/). Two potential FXREs, named α1 [nucleotides (nt) –1375/–1363 relative to the transcription start site] and α2 (nt –1295/–1283) were identified within the OSTα promoter (Fig. 4, A and C). In the case of the OSTβ promoter, a single putative FXRE (β; nt –4641/–4629), was identified (Fig. 4, B and C). All three putative FXREs in human OST promoters are in the configuration of IR-1 and exhibit a high degree of homology with the FXRE present in the human I-BABP promoter (Fig. 4C), a well-known target for activation by FXR (9).

FXR induces OSTα and OSTβ promoter activity in a ligand-dependent manner. To examine whether FXR activates OSTα and OSTβ promoters in Luc reporter gene assays, we PCR cloned the 5'-regulatory regions of both genes, so that the putative FXREs identified above were contained within the cloned sequences. The PCR products were subcloned into the pGL3basic reporter vector to generate the OSTα (–1475/+161)Luc and OSTβ (–4748/+29)Luc reporter plasmids. Huh7 cells were transiently transfected with these promoter constructs together with expression plasmids for FXR and/or RXRα. Transfected cells were treated with either 200 nM GW4064 or the vehicle DMSO. The FXR agonist GW4064 had little effect on the activity of either the OSTα or OSTβ promoter construct when the empty vector or the expression vector for RXRα alone, without FXR, was cotransfected (Fig. 5, A and B). In contrast, exogenous expression of FXR together with RXRα led to potent GW4064-dependent induction of both OSTα and OSTβ promoters. Similar results were obtained when the bile acid CDCA was used instead of the specific FXR ligand GW4064 (data not shown). Exogenous expression of FXR alone, without RXRα, led to only moderate GW4064-dependent induction of either promoter, suggesting that the FXR-RXRα heterodimer transactivates the two promoters. It may be that sufficient amounts of RXRα are endogenously expressed in Huh7 cells to sustain a low level of heterodimerization, explaining the moderate level of induction of OSTα and OSTβ promoters in the absence of exogenous RXRα expression.

FXR-RXRα heterodimers bind to IR-1 motifs in human OST promoters. To assess whether FXR-RXRα heterodimers can directly bind to IR-1 elements within human OST promoters,
EMSAs were performed using in vitro translated FXR and RXRα proteins together with 32P-labeled double-stranded oligonucleotides derived from OST promoters (Table 1). A specific DNA-protein complex formed using radioactive probes that correspond to either of the two putative FXREs from the OSTα promoter or to the single putative FXRE from the OSTβ promoter in the presence of both FXR and RXRα (Fig. 6, A–C, lane 3). When only FXR or RXRα protein was included in the binding reaction, no such complex was formed, confirming that heterodimerization of the two nuclear receptors is required for DNA binding (Fig. 6, A–C, lanes 1 and 2). The addition of FXR- and RXRα-specific antibodies resulted in supershifted complexes of retarded mobility, confirming the identity of the proteins (Fig. 6, A–C, lane 4). As a further demonstration of specificity, we performed competition experiments using 50-fold molar excesses of unlabeled oligonucleotides. The competitor oligonucleotide harboring the consensus FXRE derived from the human I-BABP promoter (Fig. 4C) efficiently abolished the interaction of the FXR-RXRα heterodimer with all three probes encompassing the FXREs from the two human OST promoters (Fig. 6, A–C, lane 5). Similarly, an excess of unlabeled oligonucleotides containing wild-type FXREs efficiently prevented the formation of DNA-protein complexes on the corresponding radiolabeled probes (Fig. 6, A–C, lane 6). In contrast, mutated oligonucleotides containing base changes predicted to disrupt FXR-RXRα DNA binding (Table 1) failed to affect protein complex formation on the radiolabeled probes (Fig. 6, A–C, lane 7). The α2-FXRE of the OSTα promoter binds the FXR-RXRα heterodimer less strongly than the α1-element (Fig. 6, A and B). This reflects the lower identity of the α2-element with the consensus FXRE, as present, for instance, in the human I-BABP promoter (Fig. 4C).

In EMSAs using nuclear extracts prepared from Huh7 cells together with FXR- and RXRα-specific antibodies, we confirmed that FXR-RXRα heterodimers endogenously present in these cells can bind to all three IR-1 elements of human OST promoters (Fig. 6, D–F).

IR-1 motifs mediate FXR-dependent induction of human OST promoters. We next investigated whether the two IR-1 elements within the human OSTα promoter and the single IR-1 element within the human OSTβ promoter functionally mediate induction by ligand-activated FXR. Point mutants were created within the IR-1 motifs in the context of the Luc-linked promoters. Huh7 cells were transfected with these mutated promoter variants in parallel with wild-type constructs. The point mutations used in this functional assay were identical to those shown to abolish DNA binding by FXR-RXRα in EMSA competition assays (Fig. 6, A–C, lane 7). In the case of the OSTα promoter, the mutation of either the α1-IR-1 alone or α1-IR-1 and α2-IR-1 elements in combination led to complete suppression of FXR-dependent induction (Fig. 7A). Mutation of the α2-IR-1 element alone also resulted in clearly decreased
ligand-dependent induction of the \textit{OST}α promoter, although to a lesser degree than the mutation of the \alpha{}1-element. This is consistent with the \alpha{}1-IR-1 motif being a stronger binding site for the FXR-RXRα heterodimer in vitro (Fig. 6, A and B). The point mutation of the single IR-1 \beta{}-element within the human \textit{OST}β promoter completely abolished inducibility by FXR in the presence of GW4064 (Fig. 7B). Taken together, in addition to directly interacting with FXR-RXRα heterodimers, the IR-1 elements identified in this study functionally mediate the induction of human \textit{OST} promoters by ligand-activated FXR.

DISCUSSION

This study identifies the heterodimeric transporter protein complex \textit{OST}α/\textit{OST}β as a target for FXR-mediated gene induction. Endogenous \textit{OST}α/\textit{OST}β mRNA levels in human hepatoma cell lines were increased after they were exposed to the bile acid CDCA or the synthetic FXR ligand GW4064 (Fig. 1). Importantly, both \textit{OST} mRNA levels were similarly elevated \textit{ex vivo} in biopsy tissue derived from the human ileum upon treatment with the bile acid CDCA (Fig. 2). Inhibition of FXR expression by siRNAs abolished the induction of \textit{OST}/

\textit{OST}β mRNAs by CDCA or GW4064 in hepatoma cell lines (Fig. 3). Binding sites for the FXR-RXRα heterodimer were identified in the \textit{OST}α gene at \textit{nt} −1375/−1363 (\alpha{}1) and −1295/−1283 (\alpha{}2) and in the \textit{OST}β gene at \textit{nt} −4641/−4629 (\beta{}). Luc reporter constructs of \textit{OST}α and \textit{OST}β promoters that contained these FXREs were transactivated by GW4064 in Huh7 cells cotransfected with FXR and RXRα expression plasmids (Fig. 5). All three sites were shown to specifically bind the FXR-RXRα heterodimer in EMSAs (Fig. 6), with the \alpha{}1-motif forming a more prominent DNA-protein complex in vitro than the \alpha{}2-motif. Site-directed mutagenesis of the \alpha{}1- and \beta{}-motifs abolished transactivation of \textit{OST}α and \textit{OST}β promoters, respectively, by GW4064, whereas mutagenesis of the \alpha{}2-motif alone led to reduced inducibility of the \textit{OST}α promoter construct in the presence of an intact \alpha{}1-motif (Fig. 7).

The data reported here add human \textit{OST}α and \textit{OST}β genes to the spectrum of FXR-regulated bile acid transporter genes within the enterohepatic circulation. The \textit{OST}α/\textit{OST}β protein complex probably represents a major efflux mechanism by which bile acids are transported from intestinal enterocytes into portal blood (4). It is thus functionally analogous to BSEP, the major bile acid efflux pump of hepatocytes. The BSEP gene is also transactivated by the nuclear bile acid receptor FXR, which binds to an IR-1 element within the BSEP promoter (1, 20, 22). Thus bile acids can induce their own efflux from enterocytes and hepatocytes through feedforward induction of the respective efflux pumps, \textit{OST}α/\textit{OST}β and BSEP. The mechanism of induction, FXR-mediated transcriptional activation, is similar for all three genes. It is intriguing that although human \textit{OST} genes do not exhibit overall sequence similarity...
and are located on separate chromosomes (the OSTα gene on chromosome 3 and the OSTβ gene on chromosome 15), they are both similarly induced through direct binding of FXR to 5′-regulatory sequences. This finding underlines the physiological importance of FXR in controlling OSTα/OSTβ expression levels.

The fact that both OST genes are positively regulated by bile acids is in accordance with the finding that levels of both Ostα and Ostβ mRNAs were increased in the cecum and proximal colon and decreased in the ileum of mice that do not express the ileal bile acid transporter Asbt (4). Mice lacking Asbt have reduced uptake of bile acids by enterocytes of the terminal ileum, resulting in decreased intracellular levels of FXR ligands and consequently reduced expression of Ostα/Ostβ. Spillover of bile acids into the colon results in bacterial deconjugation and passive absorption by colonic enterocytes, where the increased intracellular bile acid load activates FXR and induces Ostα/Ostβ gene transcription. In normal mice, Ostα and Ostβ mRNA expression closely parallel one another, and intestinal expression is highest in the ileum (4), the main site of bile acid absorption. The other putative bile acid export system expressed at the basolateral enterocyte membrane, Mrp3, is more abundantly expressed in the liver, stomach, duodenum, and proximal colon than in the ileum, making its physiological role as a main transporter of bile acids from enterocytes into portal blood questionable (2).

FXR plays a predominant role in the regulation not only of bile acid efflux systems, such as OSTα/OSTβ, BSEP, and MRP2 (10), but also of bile acid uptake. Expression of major bile acid uptake systems in enterocytes (ASBT) and hepatocytes [Na+-taurocholate cotransporting polypeptide (NTCP)] is transcriptionally repressed by bile acids through a pathway
that involves FXR-mediated induction of the repressor protein SHP (23). SHP negatively interferes with the activation of the human NTCP gene by GR (5) and of the human ASBT gene by GR and the retinoic acid receptor (RAR)-RXRα heterodimer (5, 18). Within intestinal enterocytes, the gene encoding the cytosolic bile acid binding protein I-BABP is directly transactivated by FXR through an IR-1 element in the I-BABP promoter (9), although the exact physiological role of I-BABP in intestinal bile acid absorption is unclear. The crucial role for FXR in regulating genes encoding bile acid transporters is supported by studies in FXR-null mice, which have reduced Bsep expression in hepatocytes and undetectable I-babp expression in the ileum (26). Although the intestinal expression of Ostα/Ostβ has not been measured in FXR−/− mice, it is likely that, in contrast to wild-type mice, neither transcript is inducible by feeding a diet rich in cholic acid.

In conclusion, we propose the scheme shown in Fig. 8, where the intracellular transport and basolateral efflux of bile acids from enterocytes into portal blood is induced via direct transactivation of I-BABP and OST genes by the FXR-RXRα nuclear receptor heterodimer. In contrast to the efflux of bile acids across the basolateral membrane, the Na+-dependent uptake of bile acids across the luminal enterocyte membrane is subject to feedback inhibition via FXR-mediated induction of the transcriptional repressor SHP. SHP negatively interacts with the transcriptional activators of the human ASBT gene, GR and RAR-RXRα. Via the bile acid and FXR-mediated regulation of Ostα/Ostβ expression levels described here, as well as of ASBT expression, enterocytes may coordinate the rates of bile acid efflux and uptake in response to the frequent changes in luminal bile acid load.

Fig. 7. IR-1 elements identified within hOSTα and hOSTβ promoters are functional FXREs. Huh7 cells were transfected with Luc-linked hOSTα promoter constructs harboring either WT or mutated α1-IR-1 (MUT1) and/or α2-IR-1 (MUT2) elements (A) or hOSTβ promoter constructs harboring either the WT or mutated β-IR-1 element (B). Expression vectors for FXR and/or RXRα were cotransfected as indicated. Twelve hours after transfection, cells were treated for a further 24 h with 200 nM GW4064 or the vehicle DMSO. For each reporter construct, the relative Luc activities obtained for pcDNA3.1-transfected cells treated with the vehicle DMSO are set to 1, and the fold activities in other test conditions are shown relative to this. Introduction of the mutations into IR-1 elements had no effect on basal activities of either of the OST promoter constructs.

Fig. 8. Schematic model for the role of FXR in regulating bile acid transporter genes expressed in human ileum. Human I-BABP (hI-BABP) is shown in association with the human apical sodium-dependent bile acid transporter (hASBT), as previously suggested (11). RAR, retinoic acid receptor.
ACKNOWLEDGMENTS

Dr. David Mangelsdorf and Dr. Daniel Berger are acknowledged for donating the FXR and RXR-α expression constructs and the compound GW4064, respectively. We thank Christian Hiller and Marianne Keller for excellent technical assistance. Prof. Michael Fried is acknowledged for support.

GRANTS

This study was supported by Swiss National Science Foundation Grant PPO0B-108511/1 (to S. R. Vavricka), by a research grant from the University of Zurich (to S. R. Vavricka), and a grant from the Novartis Foundation for Biomedical Research (to S. R. Vavricka).

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