Farnesoid X Receptor Activates Transcription of the Phospholipid Pump MDR3*

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The human multidrug resistance gene MDR3 encodes a P-glycoprotein that belongs to the ATP-binding cassette transporter family (ABCB4). MDR3 is a critical translocator for phospholipids across canalicular membranes of hepatocytes, evidenced by the fact that human MDR3 deficiencies result in progressive familial intrahepatic cholestasis type III. It has been reported previously that MDR3 expression is modulated by hormones, cellular stress, and xenobiotics. Here we show that the MDR3 gene is trans-activated by the farnesoid X receptor (FXR) via a direct binding of FXR/retinoid X receptor α heterodimers to a highly conserved inverted repeat element (a FXR response element) at the distal promoter (~1970 to ~1958). In FXR trans-activation assays, both the endogenous FXR agonist chenodeoxycholate and the synthetic agonist GW4064 activated the MDR3 promoter. Deletion or mutation of this inverted repeat element abolished FXR-mediated MDR3 promoter activation. Consistent with these data, MDR3 mRNA was significantly induced by both chenodeoxycholate and GW4064 in primary human hepatocytes in time- and dose-dependent fashions. In conclusion, we demonstrate that MDR3 expression is directly up-regulated by FXR. These results, together with the previous report that the bile salt export pump is a direct FXR target, suggest that FXR coordinately controls secretion of bile salts and phospholipids. Results of this study further support the notion that FXR is a master regulator of lipid metabolism.

ATP-binding cassette (ABC)† transporters constitute a large family of proteins, and many have been shown to be involved in lipid transport. MDR3 (ABCB4), a P-glycoprotein, is predominantly expressed in the liver (1) and is the critical translocator for phospholipids across canalicular membranes of hepatocytes (2). The MDR3 function is essential for the liver as evidenced by the fact that MDR3 deficiencies in humans result in progressive familial intrahepatic cholestasis type III (3, 4). A number of factors, such as hormones, cellular stress, and xenobiotics have been shown to modulate MDR3 expression (5–7). However, the underlying molecular mechanisms for MDR3 gene regulation are unclear. In this study, we demonstrate that the bile acid receptor FXR directly regulates expression of MDR3.

FXR is a nuclear receptor for bile acids and regulates expression of a number of genes in which products are critically important for bile acid and cholesterol homeostasis (8–11). FXR functions as a heterodimer with the 9-cis-retinoic acid receptor (RXRα) (12, 13), and the FXR/RXRα heterodimer activates gene transcription via binding to a specific DNA sequence comprised of two inverted hexamer repeats separated by one nucleotide (IR-1) in the target promoter. To date, there is only one reported case in which FXR down-regulates apolipoprotein A-I expression via a FXR monomer or homodimer binding to an IR-1 (14).

Previous studies (15, 16) have shown that agonist-bound FXR directly regulates expression of the bile salt export pump (BSEP), an ABC transporter (ABCB11) in which deficiencies also cause progressive familial intrahepatic cholestasis (type II) (3). We hypothesized that FXR might also regulate expression of MDR3 to coordinately control secretion of bile acids and phospholipids.

To determine whether the MDR3 gene is a target of FXR, we first treated primary human hepatocytes with FXR agonists and analyzed endogenous MDR3 expression by real time PCR and by Northern blot analysis. Indeed, in primary human hepatocytes, the MDR3 mRNA was strongly induced by both the endogenous FXR agonist CDCA and the synthetic ligand GW4064. We further identified an IR-1 element in the MDR3 promoter and demonstrated that the FXR/RXRα heterodimer specifically bound to the IR-1 and that the integrity of this IR-1 is essential for FXR-mediated promoter activation. These results indicate that the human MDR3 gene is directly trans-activated by FXR via the IR-1 element in the MDR3 promoter and suggest that FXR regulates expression of MDR3 and BSEP coordinately by a feed-forward mechanism to facilitate hepatic export of bile acids and phospholipids. These results support the critical role of FXR in cholesterol and bile acid metabolism.

MATERIALS AND METHODS

Reagents—The following reagents were obtained from Invitrogen: DMEM and Opti-MEM I medium, regular fetal bovine serum (FBS) and charcoal-stripped FBS (CS-FBS), TRizol reagents, T4 polynucleotide kinase, PCR Supermix, and oligonucleotide primers for gene cloning.
[γ-32P]ATP (3000 mCi/mmol) was obtained from Amersham Biosciences. The T7 T7 quick-coupled transcription/translation kit and reagents for β-galactosidase and luciferase assays were purchased from Promega (Madison, WI). The QuickChange mutagenesis kit was obtained from Stratagene. FuGENE 6 transfection reagent was obtained from Roche Diagnostics. CDCA was obtained from Steraloids, Inc. (Newport, RI). TaqMan reagents for CDNA synthesis and real time PCR and TaqMan oligonucleotide primers and probes for human MDR3 were purchased from Applied Biosystems (Foster City, CA).

Sequence Analysis of MDR3 Genomic Region—Previous study has identified multiple transcription start sites for human MDR3 (17). To better define the transcription start site of MDR3, we blasted the predominant form of the gene, NM_000443, against human mRNA and EST data bases. Multiple transcripts of human MDR3 were identified and retrieved. Each of them was mapped onto the Celera human genome (R26) using BLAT (18). The map indicated two transcription start sites >5 kb apart. Based on information from the full-length transcripts, we refer to the upstream site as the transcription start site of human MDR3. The genomic region spanning MDR3 and its 10-kb upstream flanking region was then extracted. Putative binding sites for transcription factors were identified by scanning this genomic sequence against the TRANSFAC data base (19) and the position-weighted matrices constructed internally.

MDR3 Promoter and Reporter Plasmid Constructs—Comparison of human MDR3 cDNA sequence by the BLAST search identified the genomic region spanning MDR3 and its 10-kb upstream-flanking region. The fragment of ~2201/+37 was amplified by PCR, and the MDR3-2201/Luc construct was generated by subcloning of the PCR-amplified fragment (~2201/+37) into the pGL3-enhancer plasmid vector (Promega) at NheI/HindIII sites. Similarly, the MDR3-2200/Luc and MDR3-2208/Luc constructs were also generated by subcloning of the PCR-amplified fragment (~2200/+37) and (~2208/+37) into the pGL3-enhancer plasmid, respectively. The sequence integrity for all constructs was confirmed by DNA sequencing. The expression vector pcDNA3.1-FXR, pcDNA3.1-RXRα, and pCMV-lacZ were described previously (20).

MDR3 Promoter Mutants—FXRRE in the MDR3 promoter was mutated using the QuickChange mutagenesis kit (Stratagene). PCR reactions were carried out according to the manufacturer’s directions with the MDR3-2200/Luc construct as a template. The sense primer was 5′-AGTTAGTAAATACATTATAGCCCTAGTGG-3′, and the antisense primer was 5′-CACTAAGGCCCATACTTTTATTCTGAT-3′ (altered bases are in bold and underlined).

Electrophoretic Mobility Shift Assay (EMSA)—cDNA encoding human FXR or RXRα was transcribed and translated using the T7 quick-coupled transcription/translation system (Promega) according to the instructions of the manufacturer. Double-stranded oligonucleotide probes for the EMSA were end-labeled with [γ-32P]ATP (3000 mCi/mmol) by T4 polynucleotide kinase. The EMSA was performed as described previously (21) with minor modifications. Briefly, 2 µl of in vitro translated FXR or RXR protein alone or together were added to 20 µl of reaction containing 10 µl Tris (pH 8.0), 40 µl KCl, 0.05% Nonidet P-40, 6% glycerol, 1 µl dithiothreitol, 1 mM sodium pyruvate, and 5 mM HEPES. For determination of gene specific expression by TaqMan analysis, cells were seeded in 6-well plates at a density of 2 million cells/well in 6-well plates were treated with 60 µM CDCA (batched bars) or 5 µM GW4046 (solid bars) at various time points (A) or with various concentrations of CDCA (B) for 24 h in DMEM containing 0.5% CS-FBS. Total RNA was prepared, and MDR3 mRNA was analyzed by TaqMan PCR as described under “Materials and Methods.” Results were normalized as the fold-change in the treated cells versus vehicle. Each value represents the mean ± S.D. of duplicate three determinations. C, Northern blot analysis for induction of human MDR2 mRNA by FXR agonist CDCA. Total RNA was isolated from primary human hepatocytes as described under “Materials and Methods,” and 10 µg were separated on a 1% agarose/formaldehyde gel, transferred to a nylon membrane, and hybridized to the MDR3 or β-actin radiolabeled cDNA probe as described under “Materials and Methods.”

FIG. 1. Induction of human MDR3 mRNA by FXR agonists in primary human hepatocytes. A and B, primary human hepatocytes at a density of 2 million cells/well in 6-well plates were treated with 60 µM CDCA (batched bars) or 5 µM GW4046 (solid bars) at various time points (A) or with various concentrations of CDCA (B) for 24 h in DMEM containing 0.5% CS-FBS. Total RNA was prepared, and MDR3 mRNA was analyzed by TaqMan PCR as described under “Materials and Methods.” Results were normalized as the fold-change in the treated cells versus vehicle. Each value represents the mean ± S.D. of duplicate three determinations. C, Northern blot analysis for induction of human MDR2 mRNA by FXR agonist CDCA. Total RNA was isolated from primary human hepatocytes as described under “Materials and Methods,” and 10 µg were separated on a 1% agarose/formaldehyde gel, transferred to a nylon membrane, and hybridized to the MDR3 or β-actin radiolabeled cDNA probe as described under “Materials and Methods.”

DNA isolation and Real Time Quantitative PCR—Total RNA was extracted from the treated human primary hepatocytes using the TRIzol reagent according to the manufacturer’s instructions. Reverse transcription reactions and TaqMan PCRs were performed according to the manufacturer’s instructions (Applied Biosystems). Sequence-specific amplification was detected with an increased fluorescent signal of carboxyfluorescein (reporter dye) during the amplification cycles. Amplification of human 18 S RNA was used in the same reaction of all samples as an internal control. Gene-specific mRNA was subsequently normalized to 18
S RNA. Levels of human MDR3 mRNA were expressed as the -fold difference of compound-treated cells against Me_2SO-treated cells.

Northern Blot Analysis—Isolation of total RNA was similar to that used in real time quantitative PCR. Total RNA (10 μg) was separated by electrophoresis on a 1% denaturing agarose gel with 1× formaldehyde/MOPS (Ambion, TX) and then transferred to a nylon membrane (Nytran SuPerCharge, Schleicher and Schuell). Blots were hybridized with 32P-labeled cDNA probe of the human MDR3 gene (GenBank accession number M23234, bases 536 to 1129) and then re-probed with a radiolabeled cDNA probe of the β-actin gene (Ambion, TX).

TAGMan Primers and Probes—Oligonucleotide primers and probe for human MDR3 were designed using the Primer Express program and were synthesized by Applied Biosystems. These sequences (5′–3′) are as follows: forward primer (GTA CTTGGTGCACTTTTCTACAGGCT), probe (6FAM-CACAGATGCTGCCCAAGTCCAAGGATAMRA), and reverse primer (TGGAAATTAGAGAACCACCTGGT) (where 6FAM is 6-carboxyfluorescein and TAMRA is carboxytetramethylrhodamine). The primers and probe for human 18S RNA were also purchased from Applied Biosystems.

RESULTS

Endogenous Expression of MDR3 Is Increased by FXR Agonists in Primary Human Hepatocytes—To determine whether MDR3 expression is regulated by FXR, primary human hepatocytes were treated with the endogenous FXR agonist CDCA (60 μM) and the synthetic FXR agonist GW4064 (5 μM) for 3, 6, 12, 24, and 48 h, and the endogenous expression of MDR3 was determined by real time PCR (TagMan). Up-regulation was readily detectable within 3 h with a maximum induction of 1.5–2-fold, which increased to around 3-fold at 6 h and about 4-fold at 12 h (Fig. 1A). Twenty-four-hour treatment yielded a peak induction of 5–6-fold, which decreased slightly at 48 h (Fig. 1A). The fact that MDR3 up-regulation by FXR agonists was readily detectable as early as 3 h suggests MDR3 is a direct target of FXR. Upon 24-h treatment, MDR3 mRNA was increased by CDCA in a dose-dependent manner with a maximum induction of 15-fold (Fig. 1B). Compared with the result in Fig. 1A, the more robust induction on MDR3 mRNA by CDCA in Fig. 1B may be because of the variation of individual donors.

Northern blot analysis was carried out to confirm the results obtained from the real time PCR. Treatment of the primary human hepatocyte cells with 60 μM CDCA or 5 μM GW4064 resulted in a 10–20-fold increase of the 4.2-kb RNA band seen with a radiolabeled human MDR3 cDNA probe (Fig. 1C). The Northern blot analysis confirms that endogenous expression of MDR3 is induced by FXR agonists.

The MDR3 Promoter Contains an IR-1 That Binds Specifically to the FXR/RXRα Heterodimer—The FXR/RXRα heterodimer binds to specific DNA sequences in promoters of target genes to regulate gene transcription. The DNA sequences recognized by the heterodimer comprise an inverted repeat separated by a single nucleotide (IR-1). A data base search using the IR-1 consensus sequence identified a highly conserved IR-1 element in the distal promoter of MDR3 (~1970 to ~1958). To examine whether the FXR/RXRα heterodimer binds to this IR-1 element, an electrophoretic mobility shift assay was performed using 32P-labeled IR-1 from the human MDR3 promoter in the presence of in vitro translated human FXR and/or human RXRα proteins. The results of EMSAs are shown in Fig. 2A. Neither FXR nor RXRα alone bound to the probe (lanes 1 and 2). However, when both FXR and RXRα proteins were present, a complex was formed indicating that it is the FXR/RXRα heterodimer that is bound by the IR-1 element (lane 3). Competition analysis showed that an unlabeled IR-1 oligonucleotide from MDR3 promoter (Fig. 2B, WT) at a 20-, 50-, or 200-fold molar excess was able to compete for binding in a dose-dependent manner (lanes 4–6), whereas the same molar excess of a mutated oligonucleotide (Fig. 2B, Mut) failed to compete for binding (lanes 7–9). Moreover, an ideal IR-1 sequence (Fig. 2B, IR-1) efficiently competed for binding at a 20-, 50-, or 200-fold molar excess (lanes 10–12). These results indi-
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Fig. 3. Deletion-mutation analysis of the human MDR3 promoter. A, schematic presentation of different MDR3 promoter constructs. The constructs of MDR3_2201-Luc and MDR3_2000-Luc contain the IR-1 sequence localized between −1970 and −1958. MDR3_1995-Luc lacks the IR-1 element, and MDR3mut-Luc contains a mutated IR-1 (mutation in lowercase type), which is otherwise the same as MDR3_2000-Luc. B and C, HepG2 cells (3.2 × 10⁴ cells/well of 96-well plates) were co-transfected with pCMV-lucZ, FXR/RXRα expression vectors, and each of the MDR3 promoter constructs as indicated in A. Cells were then treated with 50 μM CDCA (b, hatched bars) or 0.5 μM GW4064 (C, solid bars) for 40–48 h, and the cell lysate was used for determination of luciferase and β-galactosidase activities as described under “Materials and Methods.” Luciferase activities were normalized to β-galactosidase activities individually for each well. Each value represents the mean ± S.D. of six determinations. DMSO (open bars). MeSO₃.

cate that the IR-1 element in the MDR3 promoter is an authentic FXR/RXRα-binding cis element.

The IR-1 Element in Human MDR3 Promoter Is Necessary for FXR/RXRα-mediated Promoter Activation—To determine whether the IR-1 element is necessary for FXR/RXRα-mediated MDR3 promoter activation, a 2238-bp fragment of the MDR3 promoter (−2201 to +37) was cloned upstream of a luciferase reporter gene (Fig. 3A). This construct (MDR3_2201-Luc) was transiently transfected into HepG2 cells together with FXR and RXRα expression vectors, and the transfected cells were treated with 50 μM CDCA or 0.5 μM GW4064. Luciferase activity was induced by CDCA (Fig. 3B) or GW4064 (Fig. 3C) by 4–6-fold compared with the MeSO₃ control.

To further define the importance of IR-1 element in MDR3 promoter activation, two reporter constructs, MDR3_2000-Luc and MDR3_1995-Luc, were also generated (Fig. 3A). MDR3_2000-Luc is a minimal promoter that contained the IR-1 element, whereas MDR3_1995-Luc contained a deletion of IR-1 and upstream sequences (Fig. 3A). HepG2 cells were transfected with each of the constructs along with FXR and RXRα expression vectors. Luciferase activity was readily induced by 50 μM CDCA or 0.5 μM GW4064 (−5-fold) for the MDR3_2000-Luc construct, whereas only minimal induction was observed for MDR3_1995-Luc, which was similar to that of the pGL3 vector control (Fig. 3, B and C). These results indicate that the IR-1 element is necessary for FXR ligand-induced MDR3 promoter activation.

Mutation of MDR3 IR-1 Abolishes Promoter Trans-activation by FXR/RXRα—To demonstrate further that the IR-1 element at −1970 to −1958 is responsible for the trans-activation of MDR3 promoter, mutations in both halves of the IR-1 element (ATGTCAATAACCT to AaaTCAATAAAttT; mutated bases in lowercase type) were created in the MDR3_2000-Luc construct using site-directed mutagenesis, and the mutant construct (MDR3mut-Luc) was transfected in HepG2 cells together with FXR and RXRα expression vectors. Compared with MDR3_2000-Luc, MDR3mut-Luc showed only a modest residual induction by 50 μM CDCA (Fig. 3B) and 0.5 μM GW4064 (Fig. 3C). This induction was indistinguishable from that of the construct lacking the IR-1 element (MDR3_1995-Luc) and the pGL3 vector control (Fig. 3, B and C). This result indicates that the integrity of the IR-1 element in the MDR3 promoter is essential for the promoter trans-activation by FXR/RXRα.

GW4064 Activates the MDR3 Promoter in a Dose-dependent Fashion—To determine the dose dependence of MDR3 promoter activation by FXR, the MDR3_2000-Luc construct (a minimal promoter containing the IR-1 element) was transiently transfected into HepG2 cells together with the FXR and RXRα expression vectors, and cells were treated by the synthetic FXR ligand GW4064. Fig. 4 indicates that GW4064 increased the luciferase activity in a dose-dependent manner with an EC₅₀ value of 25 nM and a maximum induction of 8–10-fold. The EC₅₀ value in this assay correlates well with the binding affinity of this ligand on FXR (23). In the absence of exogenous FXR/RXRα (without co-transfection with the FXR and RXRα expression vectors), GW4064 did not significantly increase the luciferase activity (Fig. 4B). This is presumably because of a low basal level of FXR expression in HepG2 cells that was not enough for activating the MDR3 promoter construct that was transiently transfected into the cells.

The FXR and RXRα Ligands Additively Activate the MDR3 Promoter—It has been shown previously that several FXR targets are regulated by both bile acids and the RXRα ligand, 9-cis-retinoic acid (RA) (21, 23). To determine whether the MDR3 promoter is also regulated by the RXRα ligand, the MDR3_2000-Luc construct was transiently transfected into HepG2 cells together with the FXR and RXRα expression vectors. As expected, 9-cis-RA alone efficaciously increased the
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Luciferase activity in a dose-dependent manner with a maximum induction of 6-fold (Fig. 5). In the presence of 50 nM GW4064, this induction was further increased by 2–3-fold compared with that induced by 9-cis-RA alone (Fig. 5) indicating that FXR and RXRα ligands work additively on the activation of the MDR3 promoter. These results confirm that FXR is a permissive receptor and that it is the FXR/RXRα heterodimer that trans-activates the MDR3 promoter.

**DISCUSSION**

FXR is a bile acid sensor and plays a critical role in expression of hepatic transporters for the uptake and secretion of bile salts, organic anions, and other endo- or xenobiotics. Activated FXR suppresses the expression of Na+-dependent taurocholate-co-transporting polypeptide (24), a transporter that is located at the basal membrane for hepatic uptake of bile salts and organic anions. FXR increases transcription of MRP2 (25) and BSEP (15, 16), two transporters that are located at the apical membrane and are responsible for hepatic secretion of bile salts and organic anions. BSEP is critically important for the transport of bile salts across the hepatocyte canalicular membrane (26, 27). Similar to MDR3 deficiencies, BSEP mutations in humans also result in progressive familial intrahepatic cholestasis (3) suggesting that the secretion of both bile acids and phospholipids is critical for normal liver function. Previous studies have shown that FXR/bile acids induce BSEP expression, and this induction is mediated through an IR-1 (a FXR binding site) in the BSEP promoter (15, 16). We therefore hypothesized that FXR/bile acids might also increase expression of the phospholipid transporter MDR3 to coordinate the secretion of bile acids and phospholipids.

Treatment of primary human hepatocytes with the endogenous FXR agonist CDCA and the synthetic agonist GW4064 greatly induced MDR3 mRNA (Fig. 1). Consistent with this observation, an IR-1 was identified in the distal promoter of MDR3. Furthermore, deletion or mutation of this IR-1 abolished the binding by the FXR/RXR heterodimer and also abolished MDR3 promoter activation (Figs. 2 and 3) indicating that it is this IR-1 that mediates the up-regulation of MDR3 by FXR. These observations support the idea that bile acids may increase phospholipid secretion via FXR up-regulation of MDR3.

Although many of the IR-1 elements of known FXR target genes are located in the proximal promoter, it is not uncommon that the FXRE of human MDR3 promoter is about 2 kb upstream of the transcription start site. It has been shown that the apolipoprotein C-II promoter contains a functional FXRE that is located 7.5 kb upstream of the transcription start site and that this FXRE is essential for FXR-mediated up-regulation of apolipoprotein C-II (28).

The discovery of up-regulation of MDR3 by FXR makes good physiological sense. When bile acid concentrations in the liver are elevated, activated FXR on one hand increases expression of BSEP for promoting bile salt secretion and on the other hand up-regulates MDR3 expression resulting in an increased phospholipid secretion. The coordinated regulation of MDR3 and BSEP by FXR represents a feed-forward mechanism for hepatic export of bile acids and phospholipids. The increased expression of BSEP and MDR3 would lead to the efflux of bile acids and phospholipids into bile canaliculi, although both transporters may also influence the secretion of cholesterol into bile resulting in an appropriate ratio of bile acids/phospholipid/cholesterol. This study provides further evidence for the critical role of FXR in bile acid and cholesterol metabolism.

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