 Syndecan-1 Expression Is Regulated in an Isoform-specific Manner by the Farnesoid-X Receptor*

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Syndecan-1 (SDC1), a transmembrane heparan sulfate proteoglycan that participates in the binding and internalization of extracellular ligands, was identified in a screen designed to isolate genes that are regulated by the farnesoid X-receptor (FXR, NR1H4). Treatment of human hepatocytes with either naturally occurring (chenodeoxycholic acid) or synthetic (GW4064) FXR ligands resulted in both induction of SDC1 mRNA and enhanced binding, internalization, and degradation of low density lipoprotein. Transient transfection assays, using wild-type and mutant SDC1 promoter-luciferase genes, led to the identification of a nuclear hormone receptor-binding hexad arranged as a direct repeat separated by one nucleotide (DR-1) in the proximal promoter that was necessary and sufficient for activation by FXR. The wild-type, but not a mutated DR-1 element, conferred FXR responsiveness to a heterologous thymidine kinase promoter-reporter gene. Four murine FXR isoforms have been identified recently that differ either at their amino terminus and/or by the presence or absence of four amino acids in the hinge region. Interestingly, the activities of the human SDC1 promoter-reporter constructs were highly induced by the two FXR isoforms that do not contain the four-amino acid insert and were unresponsive to the isoforms containing the four amino acids. Thus, current studies demonstrate that hepatic SDC1 is induced in an FXR isorprotein-specific manner. Increased expression of SDC1 may account in part for the hypotriglyceridemic effect that can result from the administration of chenodeoxycholic acid to humans.

Nuclear hormone receptors are a superfamily of ligand-activated transcription factors that play critical roles in development and adult physiology (1). Most family members function as heterodimers with a common partner, the 9-cis-retinoic acid receptor (RXR)† (2). A number of these nuclear receptors have been shown to undergo a conformational change upon binding ligand that promotes the release of co-repressor proteins and the subsequent recruitment of co-activator proteins; the net result is increased transcription of the target gene (3, 4). The farnesoid-X receptor (FXR, NR1H4) is a member of this sub-class and binds as an FXR/RXR heterodimer to FXR-response elements (FXREs) (5, 6). In 1999, three groups independently reported (7–9) that specific bile acids bind to and activate both human and rat FXR at physiological concentrations. The most potent of these natural ligands is the primary bile acid chenodeoxycholic acid (CDCA). These unexpected results extended our understanding of bile acids beyond their traditional roles in the solubilization and absorption of dietary lipids and fat-soluble vitamins to a new class of hormonal ligands that play key roles in gene expression.

Recently, four murine and human FXR isoforms (FXRα1, α2, β1, and β2) were identified that are derived from a single gene as a result of alternative promoter usage and alternative splicing of the mRNA (10, 11). The FXRα isoforms have an extended amino terminus with respect to the FXRβ isoforms. In addition, FXRα2 and FXRβ2 contain a four-amino acid insert in the hinge region that is absent from both FXRα1 and FXRβ1 (10, 11). The four isoforms are both differentially expressed in a tissue-specific manner and differentially activate the FXR target gene encoding the intestinal bile acid-binding protein (1-BABP) (10). In contrast, all other FXR target genes studied to date (SHP, BSEP, and PLTP) are activated to similar degrees by all four FXR isoforms (10).

FXR is highly expressed in the liver, intestine, kidney, and

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†The abbreviations used are: RXR, retinoid X receptor; RXRα, retinoid X receptor α; FXR, farnesoid-X receptor; h, human; r, rat; m, murine; SDC1, syndecan-1; HSPG, heparan sulfate proteoglycan; apoC-II, apolipoprotein C-II; apoE, apolipoprotein E; CDCA, chenodeoxycholic acid; DR-n, direct repeat with n-bp spacer; IR-n, inverted repeat with n-bp spacer, ER-8, everted repeat with 8-bp spacer; EMSA, electrophoretic mobility shift assay; GW4064, 3-(2,6-dichlorophenyl)-4-(3-carboxy-2-chloro-stilben-4-yl)-oxymethyl-5-isopropyl-oxazole; 1-BABP, ileal bile acid-binding protein; SHP, small heterodimer partner; LPL, lipoprotein lipase; LDL, low density lipoprotein; mLDL, methylated LDL; PPAR, peroxisome proliferator-activated receptor; FXRE, farnesoid-X receptor-response element; mLDL, methylated low density lipoprotein; MEM, minimum Eagle's medium; PBS, fetal bovine serum; se-FBS, superstripped FBS; WT, wild type; PLTP, phospholipid transfer protein.
adrenal gland (5) and at low levels in the heart, lung, stomach, and adipose tissue (10). Targeted deletion of FXR in a murine model revealed an important role for FXR in cholesterol and bile acid homeostasis because the FXR null mouse was unable to respond normally to diets enriched in fat, cholesterol, or bile acids (12). This imbalance was presumably due to the inability of the mice to regulate the FXR target genes encoding several bile acid and organic anion transporters, including BSEP (13, 14), OATP2 (15), I-BABP (14), and SHP (NR0B2). SHP, an unusual member of the nuclear receptor superfamily that lacks a DNA binding domain, functions as a transcriptional repressor and inhibits transcription of CYP7A, a regulatory gene for bile acid synthesis (15, 16).

Other FXR target genes encode the secreted proteins phospholipid transfer protein (PLTP) (17, 18), apoE (19), and apoCII (20), all known to be involved in the metabolism of plasma lipids and lipoproteins. The observation that plasma triglyceride levels declined when rodents were treated with either a synthetic (GW4604) (21) or a natural (CDCA) FXR ligand (20) is consistent with the hypothesis that activation of FXR modulates lipoprotein levels. The observation that administration of a cholesterol-rich diet to FXR null mice results in increased serum levels of cholesterol and triglycerides and increased levels of proatherogenic lipoproteins provides additional support for this hypothesis (12). Taken together, these data suggest that activated FXR results in increased metabolism of plasma lipoproteins.

SDC1 is a member of the syndecan family of transmembrane heparan sulfate proteoglycans, which are widely expressed in partially overlapping patterns of expression in many cell types and tissues (22, 23). Their principal function appears to be to modulate the ligand-dependent activation of primary signaling receptors at the cell surface leading to an increase in binding and/or internalization of extracellular ligands (23). SDC1 is highly expressed in the liver, where heparan sulfate proteoglycans (HSPGs) bind to lipoproteins via a number of bridging proteins that include lipoprotein lipase (LPL) (24), apoE (25), and hepatic lipase (26). Several studies (27) have detailed an important role for such binding in the hepatic clearance of lipoproteins. The HSPGs on the hepatocyte cell surface are thought to bind to and sequester remnants prior to their transfer to specific receptors leading to an increase in binding and/or internalization of extracellular ligands (23). SDC1 is highly expressed in the liver, where heparan sulfate proteoglycans (HSPGs) bind to lipoproteins via a number of bridging proteins that include lipoprotein lipase (LPL) (24), apoE (25), and hepatic lipase (26). Several studies (27) have detailed an important role for such binding in the hepatic clearance of lipoprotein remnants. The HSPGs on the hepatocyte cell surface are thought to bind to and sequester remnants prior to their transfer to specific receptors such as LDL receptor and LDL receptor-related protein and subsequent endocytosis. Support for this proposal comes from the following observations: (i) apoE- or LPL-enhanced internalization of remnant-like lipoproteins is abolished by either enzymatic or genetic removal of HSPGs from the cell surface (24, 25); and (ii) in vivo injection of heparinase into the portal vein of mice reduces hepatic clearance of [125I]-labeled β-very low density lipoproteins enriched in apoE (25). A role for the independent transport of lipoprotein remnants or LDL into hepatocytes by SDC1, in the absence of other receptors, has also been described (28, 29).

Importantly, Fuki et al. (30, 31) showed that transfection of Chinese hamster ovary cells with an expression vector for SDC1 led to a significant increase in the catabolism of lipoprotein remnants enriched in LPL and that chimeric Frn-SDC1 receptors could internalize IgG in the absence of endogenous IgG receptors. However, the relative contribution of SDC1, LDL receptor, and LDL receptor-related protein to remnant/lipoprotein clearance in vivo awaits further investigation.

In the current study, we demonstrate that treatment of either primary human hepatocytes or cells derived from human hepatomas with FXR ligands results in increased expression of SDC1 mRNA and increased cellular binding, uptake, and degradation of both LDL and a preformed LDL-LPL complex. The current studies suggest that ligands for FXR increase the expression of the SDC1 gene and result in enhanced processing and clearance of lipoproteins.

**EXPERIMENTAL PROCEDURES**

**Materials**—GW4064 and LG100153 were gifts from Dr. Patrick Maloney (GlaxoSmithKline) (21) and Dr. Richard Heyman (Ligand Pharmaceuticals) (20), respectively. The retroviral vector MSCV-ires-Neo was a gift from Dr. Owen Witte (UCLA). Mammalian expression vectors for rat FXR (pCMX-rXR) and human RXRα (pCMX-hRXRα) were gifts from Dr. Ron Evans (Salk Institute, La Jolla, CA). Mammalian expression vectors for human PPARγ (pCMX-hPPARγ) and PPARα (pCMX-hPPARα) were gifts from Dr. Peter Tontonoz (UCLA). Mammalian expression vectors for murine FXR (pCMX Fxrα1, -α2, -α1, and -β2) have been described (10). Cycloheximide and actinomycin D were purchased from Sigma. The sources of other reagents have been noted elsewhere (32).

**Cell Culture and Stable Cell Lines**—The generation and maintenance of HepG2 and stably infected HepG2-FXR cells have been described (20). HuH7 cells were maintained in modified Eagle’s medium supplemented with 10% fetal bovine serum. Primary human hepatocytes were obtained from BioWhittaker (Walkersville, MD) and were cultured on Matrigel-coated 6-well plates at a density of 1.5 × 10^6 cells per well. Culture media consisted of serum-free Williams’ E medium supplemented with 100 mM dexamethasone, 100 units/ml penicillin G, 100 μg/ml streptomycin.

**RNA Isolation and Northern Blot Hybridization**—Unless otherwise indicated, HepG2 and HuH7 cells were cultured in medium containing superstriped FBS for 24 h before the addition of ligands or Me2SO (vehicle) for an additional 8–24 h. Primary human hepatocytes were cultured in growth medium with the addition of 1 μM GW4064 or Me2SO (vehicle) for 8 or 12 h. Total RNA was isolated using TRIzol reagent and was resolved (5–10 μg/lane) on a 1% agarose, 2.2 M formaldehyde gel, transferred to a nylon membrane (Hybond N+) and cross-linked to the membrane with UV light. cDNA probes were radiolabeled with [32P]dCTP using the Rediprime II labelling kit (Amersham Biosciences), and membranes were hybridized using the QuikHyb hybridization solution (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. Blots were normalized for variations of RNA loading by hybridization to a control probe, either 18 S ribosomal cDNA or the ribosomal protein 36B4. The RNA levels were quantitated using a PhosphorImager (ImageQuant software; Amersham Biosciences).

**Reporter Genes**—The human SDC1 proximal promoter (−1298 to +53) was amplified from the human BAC Clone RP11-202B22 and cloned into KpnI/VNheI sites of the pGL3 basic vector (Promega). The −1298/+53 and −937/+53 constructs contain either a wild-type or mutant DR-1 element generated using the common 5′ primer (5′-gagagggattcggctctcggagagggg-3′) and a 5′ primer with a sequence of either 5′-aagactaacggctcttcggggc-3′ or 5′-aagacggctcttcggggc-3′, which both abolish binding. The two copy reporter construct DR-1 (pTK-2xDR-1 WT) was generated by annealing the oligonucleotides 5′-gagagggctcttcggggctcttcggggctcttcggggc-3′ and 5′-gagagggctcttcggggctcttcgggg-3′. The two copy reporter construct DR-1 (pTK-2xDR-1 WT) was generated by annealing the oligonucleotides 5′-gagagggctcttcggggctcttcggggctcttcggggc-3′ and 5′-gagagggctcttcggggctcttcgggg-3′. The two copy reporter construct DR-1 (pTK-2xDR-1 WT) was generated by annealing the oligonucleotides 5′-gagagggctcttcggggctcttcggggctcttcggggc-3′ and 5′-gagagggctcttcggggctcttcgggg-3′. The two copy reporter construct DR-1 (pTK-2xDR-1 WT) was generated by annealing the oligonucleotides 5′-gagagggctcttcggggctcttcggggctcttcggggc-3′ and 5′-gagagggctcttcggggctcttcgggg-3′. The two copy reporter construct DR-1 (pTK-2xDR-1 WT) was generated by annealing the oligonucleotides 5′-gagagggctcttcggggctcttcggggctcttcggggc-3′ and 5′-gagagggctcttcggggctcttcgggg-3′. The two copy reporter construct DR-1 (pTK-2xDR-1 WT) was generated by annealing the oligonucleotides 5′-gagagggctcttcggggctcttcggggctcttcggggc-3′ and 5′-gagagggctcttcggggctcttcgggg-3′.

**Transient Transfections and Reporter Gene Assays**—HepG2 cells were transiently transfected using the MBS mammalian transfection kit (Strategene), with minor modifications. HepG2 cells, in 48-well plates, were transiently transfected with a reporter plasmid (100 ng) and either 50 ng of pCMX-rXR, pCMX-mFXR1, pCMX-mFXR2, pCMX-mFXR61, pCMX-mFXR2, VP16-hFLX, pSgS5-LSHP, pSgS5-HTR4, pCMX-PMPARα, pCMX-PMPARγ, or pCMX-hHNF4α together with 5 ng of pCMX-hRXRα and 50 ng of pCMX-β-galactosidase, as indicated in the specific figure legends. After 3.5 h, the cells were treated with 10% charcoal/dextran-treated “superstriped” FBS (ss-FBS) (HyClone, Logan, UT) and one of the following ligands: CDCA, LG100153 (synthetic RXR agonist), 3′-(2,6-dichlorophenyl)-4′-(3′-carboxy-2-chloro-stilbene-4′)- oxyphenyl-5-isopropyl-isoxazole (GW 4064). The cells were lysed, and the luciferase activities were normalized to β-galactosidase activity (32). All transfections were performed in triplicate, and similar results were obtained in at least three independent experiments.
Electromobility Shift Assays (EMSA)—EMSA were performed essentially as described elsewhere (32). FXR-mediated catabolism of lipoproteins in our system are capitalized.

Lipoprotein Binding—We relied on previously published protocols to examine proteoglycan-mediated catabolism of lipoproteins in our system (24, 35). As a model ligand, we used LPL-enriched [125I]-labeled methylated LDL (LPL/2% MLDL), which binds HSPGs but does not interact with the LDL receptor. The LPL was purified from cows’ milk by heparin-agarose chromatography (28). The [125I]-LDL was prepared from fresh human plasma by ultracentrifugation (1.019 < d < 1.063 g/ml), radioliodination, and finally reductive methylation of ~35% of the lysyl residues to abolish LDL receptor binding (28, 35). Monolayers of HepG2-FXR cells were incubated at 37°C for 8 h. The double-stranded oligonucleotides were annealed from the following single-stranded oligonucleotides and an oligonucleotide corresponding to the complementary sequence; SDC1 DR-1 WT: 5′-gatccggttctcctgagacctgtcctgagacctgtctctc-3′ or SDC1 DR-1 mut: 5′-gatccggttctccacctgagacctgagacctgtctctc-3′. Half-sites are indicated in boldface, and mutations are capitalized.

RESULTS

Induction of SDC1 by Bile Acids and the Synthetic FXR Ligand GW4064—In an effort to identify target genes that are regulated by the bile acid receptor FXR, HepG2 cells were infected with retroviral vectors that express either rat FXR or the neomycin-resistant gene or the neomycin-resistant gene alone (20). G418-resistant cells were isolated, and pooled cell populations were propagated that harbored either the vector alone (HepG2-Neo) or overexpressed FXR (HepG2-FXR). Total RNA was isolated from HepG2-Neo or HepG2-FXR cells that had been treated for 24 h with either vehicle (MeSO4), the FXR ligand CDCA (100 μM), or the synthetic FXR ligand GW4064 (1 μM). These RNA samples were then used to prepare biotinylated cRNAs that were hybridized to high-density microarrays containing ~6,000 cDNAs/ESTs (Affymetrix HuFL Gene Chip). Analysis of the microarray data identified a number of genes, including SDC1, whose mRNAs were induced by treatment of the cells with either natural or synthetic FXR ligands. We chose to explore FXR-mediated regulation of SDC1 in the absence of protein synthesis. In order to determine whether protein synthesis is required for the induction of SDC1 mRNA by FXR ligands, HepG2-FXR cells were treated with cycloheximide prior to the addition of CDCA or GW4064. Northern blot analysis illustrates that the induction of SDC1 mRNA by bile acids was the result of the activation of FXR, and not PXR, as FXR treatment of HepG2-FXR cells with rifampicin, a potent PXR ligand. The data of Fig. 2A show that neither SDC1 nor SHP mRNAs were induced by rifampicin, although this treatment resulted in induction of the FXR target gene Cyp3a11 (data not shown). Taken together, the results of Figs. 1 and 2 indicate that induction of human SDC1 mRNA levels is dependent upon activation of hepatic FXR.

FIG. 1. Induction of SDC1 mRNA by FXR ligands in HepG2-FXR cells. A, SDC1 mRNA levels are induced by FXR ligands in a dose-dependent manner. HepG2-FXR cells were treated for 24 h with increasing concentrations of CDCA (50, 100, and 250 μM or GW4064 0.1, 1, and 10 μM). Total RNA was isolated, separated on a 1% agarose/formaldehyde gel, transferred to a nylon membrane, and sequentially hybridized to radiolabeled cDNA probes for SDC1, SHP, and 36B4, as described under “Experimental Procedures.” The relative SDC1 mRNA levels are indicated.

Electromobility Shift Assays (EMSA)—EMSA were performed essentially as described elsewhere (32). mFXR were synthesized from the CMX-FXR construct, and CMX-hFXR expression vectors, respectively, using the TNT T7-Coupled Reticulocyte System (Promega, Madison, WI). Unprogrammed lysate was prepared using the pCMX vector. Binding reactions contained 10 μl HEPES, pH 7.8, 60 μM KCl, 0.2% Nonidet P-40, 6% glycerol, 2 μM dithiothreitol, 2 μg of poly(dI-dC), and 2.5 μl of each receptor protein. Control incubations received unprogrammed lysate alone. Reactions were preincubated on ice for 30 min prior to the addition of 32P-labeled double-stranded oligonucleotide probe (0.2 pmol). Competitive oligonucleotides were added to the preincubation at a 50-, 100-, or 500-fold molar excess. Samples were held on ice for a further 5 min, and the protein-DNA complexes were resolved on a pre-electrophoresed 5% polyacrylamide gel in 1× TBE (45 mM Tris-borate, 1 mM EDTA) at 4°C. Gels were dried and autoradiographed at −70°C for 8 h. The double-stranded oligonucleotides were annealed from the following single-stranded oligonucleotides and an oligonucleotide corresponding to the complementary sequence: SDC1 DR-1 WT: 5′-gatccggttctcctgagacctgtcctgagacctgtctctc-3′ or SDC1 DR-1 mut: 5′-gatccggttctccacctgagacctgagacctgtctctc-3′. Half-sites are indicated in boldface, and mutations are capitalized.

Induction of SDC1 by Bile Acids and the Synthetic FXR Ligand GW4064—In an effort to identify target genes that are regulated by the bile acid receptor FXR, HepG2 cells were infected with retroviral vectors that express either rat FXR2 and the neomycin-resistant gene or the neomycin-resistant gene alone (20). G418-resistant cells were isolated, and pooled cell populations were propagated that harbored either the vector alone (HepG2-Neo) or overexpressed FXR (HepG2-FXR). Total RNA was isolated from HepG2-Neo or HepG2-FXR cells that had been treated for 24 h with either vehicle (MeSO4), the FXR ligand CDCA (100 μM), or the synthetic FXR ligand GW4064 (1 μM). These RNA samples were then used to prepare biotinylated cRNAs that were hybridized to high density microarrays containing ~6,000 cDNAs/ESTs (Affymetrix HuFL Gene Chip). Analysis of the microarray data identified a number of genes, including SDC1, whose mRNAs were induced by treatment of the cells with either natural or synthetic FXR ligands. We chose to explore FXR-mediated regulation of SDC1 based on the fact that it has been reported to be involved in the binding and internalization of plasma lipoproteins (37). In order to confirm that SDC1 mRNA levels were induced in response to FXR ligands, HepG2-FXR cells were treated with CDCA or GW4064 for 24 h prior to RNA isolation. Northern blot assays showed that both FXR ligands resulted in a dose-dependent increase in the levels of two SDC1 mRNAs (Fig. 1). As expected, the mRNA levels for SHP, a previously characterized FXR target gene (15, 16), were highly induced in response to both ligands (Fig. 1). Induction of SDC1 and SHP mRNAs was also observed when the human hepatoma HuH7 cell line was treated with CDCA or GW4064 (Fig. 2A).

In order to extend our results to a more physiologically relevant system, we treated primary cultured human hepatocytes with GW4064. As shown by Northern blot analysis, SDC1 mRNA levels were induced 3.5-fold after 8 h of treatment (Fig. 2B). A significant, albeit less robust, induction (2.4-fold) was observed after 12 h (Fig. 2B). Induction of SHP mRNA paralleled those of SDC1 (Fig. 2B).

The pregnane-X receptor (PXR) is known to be activated by pathophysiologically relevant concentrations of bile acids (38). To confirm that the induction of SDC1 mRNA levels by bile acids was the result of the activation of FXR, and not PXR, we treated HuH7 cells with rifampicin, a potent PXR ligand. The data of Fig. 2A show that neither SDC1 nor SHP mRNAs were induced by rifampicin, although this treatment resulted in induction of the PXR target gene Cyp3a11 (data not shown). Taken together, the results of Figs. 1 and 2 indicate that induction of human SDC1 mRNA levels is dependent upon activation of hepatic FXR.

Induction of SDC1 mRNA Levels by Activated FXR Is a Primary Response—In order to determine whether protein synthesis is required for the induction of SDC1 mRNA by FXR ligands, HepG2-FXR cells were treated with cycloheximide prior to the addition of CDCA or GW4064. Northern blot analysis illustrates that the induction of SDC1 mRNA levels by FXR ligands was not blocked by cycloheximide (Fig. 3A). A similar pattern was observed for SHP, a known FXR target gene (Fig. 3A). Consequently, we conclude that the induction of SDC1 mRNA by FXR ligands is likely a primary response, as it occurs in the absence of protein synthesis.

Induction of SDC1 mRNA Levels by FXR Ligands Is Attenuated by Actinomycin D—The data of Fig. 3B demonstrate that incubation of HepG2-FXR cells for 8 h with CDCA resulted in induction of SDC1 and SHP mRNA levels by a process that was attenuated by actinomycin D, an inhibitor of RNA polymerase II.

The experiment shown in Fig. 3C was performed in order to rule out the possibility that FXR ligands induce SDC1 mRNA levels through a mechanism involving stabilization of the mRNA. HepG2-FXR cells were pretreated for 24 h with either
was isolated at the time points indicated, and the relative polymerase II-dependent transcription. Subsequently, RNA levels. Actinomycin D was then added to all cells to inhibit conditions the half-life of C, left panel 3

Previous studies (6, 32) have shown that both IR-1 and SDC1 induction of C, right panel). Based on the results of Figs. 1 and 2, we conclude that SHP mRNA levels is dependent on increased stability of the SDC1 mRNA. Under the same conditions, we determined that SHP mRNA half-life is ~4 h and is also unaffected by GW4064 treatment (Fig. 3A). Based on the results of Figs. 1–3, we conclude that induction of SDC1 mRNA levels is dependent on increased transcription of the gene in response to activated FXR.

The SDC1 Promoter Contains a Novel FXR-response Element—Previous studies (6, 32) have shown that both IR-1 and ER-8 motifs can function as FXRREs. Computer-assisted analysis of 10 kb of the published nucleotide sequence upstream of the transcriptional start site of the human SDC1 gene failed to identify any sequences corresponding to either an IR-1 or ER-8. Consequently, in order to identify a cis-acting element responsible for the observed induction of SDC1 mRNA levels by FXR ligands, we cloned ~1.3 kb of the proximal promoter of the SDC1 gene and a series of 5’ deletions into the luciferase reporter pGL3 (Promega). These reporters were co-transfected into HepG2 cells in the presence or absence of plasmids encoding RXR and either rat FXR or the constitutively active VP16-FXR fusion protein. The cells were subsequently treated for 24 h with FXR-specific ligands. In the current studies with SDC1-reporter constructs, as in previous transient transfection studies from this laboratory (20, 32), the co-transfected plasmid encoding FXR corresponded to the rat FXRα2 isoform. This isoform lacks both the amino-terminal extension that is present in the β isoform and the four-amino acid insert in the hinge region (10).

Co-transfection of a reporter construct under the control of the SDC1 promoter (nucleotides −1298 to +93 relative to the transcription start site) with FXR and RXR led to a 20-fold increase in luciferase activity following the addition of GW4064 (Fig. 4A). Co-transfection of this reporter with a plasmid encoding the constitutively active VP16-FXR showed an ~13-fold ligand-independent induction of luciferase activity (Fig. 4A). Similar results were observed with longer reporter constructs (~5 kb, data not shown), suggesting that the FXR-responsive element in the SDC1 promoter was contained within the 1246 bp flanking the 5’ end of the transcriptional start site of the SDC1 gene.

Studies with reporter genes containing 5’ deletions demonstrated that FXR-dependent induction was seen when the promoter contained nucleotides −937 to +53 (Fig. 4A) but not when the promoter contained nucleotides −888 to +53 of the SDC1 gene (data not shown). Analysis of the region between −937 and −888 (which identified a sequence (AGAGCAnAGGGGA, at −921 to −908 bps) that shows weak homology with an idealized DR-1 element. Whereas DR-1 elements with the consensus hexad sequence AGGTCA have been shown to form an in vitro complex with the FXR/RXR heterodimer (17), no DR-1 element has been shown to support regulation by FXR in vivo.

We next investigated the importance of this DR-1 element in FXR-dependent reporter activation by introducing 4 single nucleotide mutations to produce pGL3SDC1 (−937)Mut. Fig. 4A shows that induction of this mutant reporter in response to either FXR and GW4064 or VP16-FXR was greatly attenuated compared with the wild-type control reporter. The empty pGL3 control vector was unaffected by GW4064 or VP16-FXR (Fig. 4A).

The DR-1 Functions as an FXRE—To confirm that the DR-1 element identified in the SDC1 promoter is able to act as an FXRE, we constructed a luciferase promoter gene under the control of two copies of either the wild-type (pTK-2x(DR-1)WT) or mutant (pTK-2x(DR-1)Mut) element. The data show that pTK-2x(DR-1)WT luciferase reporter was activated over 50-fold in an FXR and FXR ligand-dependent manner (Fig. 4B). Co-transfection of the same reporter with the plasmid encoding VP16-FXR led to a similar increase in luciferase activity, by a process that was ligand-independent (Fig. 4B). In contrast, induction was completely abrogated when the reporter gene contained two copies of the mutant DR-1 element (Fig. 4B). As expected, co-transfection of FXR/RXR or VP16-FXR/RXR had no effect on luciferase activity when the empty pTK control vector was used (Fig. 4B).

A number of nuclear receptors, including PPARs, HNF4α, and TR4, have been shown to bind to sequences that correspond to consensus DR-1 elements and to subsequently activate transcription. However, the data of Fig. 4C demonstrate that the pTK-2x(DR-1)WT reporter gene was specifically induced by activated FXR and was unresponsive to HNF4α, TR4, or SHP, or to ligand-activated PPARs or PPARγ. Thus, of the seven nuclear receptors examined, only FXR/RXR was able to activate the reporter gene via the non-consensus DR-1 element.

To provide additional evidence that the DR-1 element in the SDC1 promoter can function as an FXRE, we performed EMSA competition assays as shown in Fig. 5. As expected, FXR/RXR

![Figure 2: Induction of SDC1 mRNA by FXR ligands in multiple cell types. A, SDC1 levels are induced in HuH7 cells by ligands for FXR but not FXR. HuH7 cells were treated for 24 h with vehicle (MeSO4) or the indicated concentrations of ligands for FXR (CDCA, GW4064, RXR (LG100153) (LG), or FXR (rifampicin). RNA was isolated and analyzed as described in the legend to Fig. 1. B, SDC1 mRNA levels in primary human hepatocytes are induced by FXR ligands. Primary human hepatocytes were treated for 8 or 12 h as indicated with 1 μM GW4064 or vehicle (DMSO). RNA was isolated and analyzed as described in the legend to Fig. 1.](image-url)
bound to a radiolabeled probe that contains the IR-1 element from the PLTP promoter (Fig. 5). Formation of this FXR/RXR/H18528 DNA complex was competed away by unlabeled PLTP IR-1 or wild-type SDC1 DR-1 but not by DNA containing the mutant DR-1 (Fig. 5). These in vitro data provide additional support for the hypothesis that the DR-1 element functions as an FXRE.

Induction of the SDC1 by FXR Is Isoform-specific—Alternate splicing and promoter usage produces four FXR isoforms from the sole FXR gene in both mouse and humans (10, 11) (data not shown). Some genes (SHP, BSEP, and PLTP) are transcriptionally activated to similar levels by all four isoforms (10). However, I-BABP is, to date, unique because it is activated in an FXR isoform-specific manner; the endogenous gene is highly induced by FXR/H92512 and FXR/H92522, which lack the four-amino acid insert in the hinge region but poorly activated by FXR/H92511 and FXR/H92521 (10).

To investigate the possibility that SDC1 levels are induced by FXR in an isoform-specific manner, we co-transfected cells with the pGL3-SDC(-937)WT reporter, RXR, and individual murine FXR isoforms, and then treated the cells with the FXR-specific ligand GW4064. As shown in Fig. 6A, luciferase activity was increased exclusively in response to the two FXR isoforms (FXR/H92512 and FXR/H92522) that do not contain the four-amino acid insert adjacent to the DNA binding domain of the receptor. Co-transfection of plasmids that encode the isoforms that contain the four-amino acid insertion (FXR/H92511 and FXR/H92521) failed to induce reporter activity in response to GW4064 (Fig. 6A).

Similar results were obtained when the pTK-2x(DR-1)WT construct was used as a reporter; luciferase activity was induced 20-fold in the presence of FXR/H92512 or FXR/H92522 and GW4064 but was unaffected in the presence of FXR/H92511 or FXR/H92521 (Fig. 6B). In contrast, consistent with our recent report (10), a reporter construct under the control of two copies of the IR-1 element from the PLTP promoter was activated by all four FXR isoforms (Fig. 6C). The empty pGL3 vector was unaffected by these treatments (Fig. 6D). Taken together, the transfection data indicate that a novel DR-1 element in the proximal promoter of the human SDC1 promoter functions as an FXRE and controls transcriptional induction of the gene. Moreover, this induction is absolutely FXR isoform-specific.

FXR Ligands Cause Increased Endocytosis of Lipoproteins—SDC1 is abundantly expressed in the adult liver (25), where HSPGs are involved in the binding and internalization of lipoproteins (24, 25). We hypothesized that the increase in SDC1 mRNA levels observed upon treatment of HepG2-FXR cells with FXR ligands would correlate with changes in the lipoprotein binding capacity of the cells. To test this hypothesis, we assayed the effect of FXR ligands on the surface binding, internalization, and degradation of [125I]-labeled methylated LDL.
Methylation is known to block the interaction of LDL with the LDL receptor (39). Pre-treatment of HepG2-FXR cells with 1 μM GW4064 for 24 h led to robust increases in the binding, internalization, and degradation of the LPL-mLDL complex (Fig. 7, A–C), consistent with an increase in SDC1 expression. Interestingly, there was also a significant increase in the binding, internalization, and catabolism of mLDL in the absence of exogenous LPL (Fig. 7). This latter observation may result from multiple transcriptional effects of FXR, which include increased expression of both SDC1 (Fig. 1) and apoE (19). These results, coupled with previous reports (12, 17, 18, 20), suggest that FXR plays an important role in multiple steps in lipoprotein metabolism by regulating the expression of several genes (PLTP, apoCII,
and apoE) involved in lipoprotein catabolism and by increasing the lipoprotein/remnant clearance potential of the hepatocyte. This increase in lipoprotein clearance correlates with the changes in SDC1 mRNA levels reported here. In contrast, neither LDL receptor-related protein nor LDL receptor mRNA levels changed following treatment with FXR ligands, as determined by either Northern blot assays or analysis of the data derived from the microarrays, respectively (data not shown).

**DISCUSSION**

The current study identifies SDC1 as a novel FXR target gene. SDC1 is a trans-membrane heparan sulfate proteoglycan that participates in the binding and internalization of a wide variety of extracellular ligands (23). It has been proposed that SDC1 functions as a co-receptor, as it binds a variety of ligands prior to their binding to co-localized receptors (23). Because expression of heparan sulfate proteoglycans on the sinusoidal membranes of hepatocytes has been shown to be important in mediating lipoprotein remnant uptake from the space of Disse (27), our data suggest that the activation of FXR is likely to enhance clearance of such remnants from the circulation.

The current studies demonstrate that SDC1 mRNA levels are highly induced in HepG2 cells, HuH7 cells, and primary human hepatocytes in response to natural and synthetic FXR ligands. Interestingly, the FXR-dependent induction of SDC1 mRNA may be specific to humans/primates, because similar studies using multiple rodent hepatic cell lines and primary murine hepatocytes failed to demonstrate regulation of Sdc1 levels by ligands for FXR (data not shown). Characterization of the induction of SDC1 mRNA levels in response to FXR ligands suggests a primary transcriptional mechanism because treatment of HepG2-FXR cells with FXR ligands and cycloheximide did not block induction of SDC1 mRNA, whereas induction was completely blocked by actinomycin D (Fig. 3). The finding that FXR ligands do not affect the half-life of the SDC1 mRNA provides additional support for the hypothesis that SDC1 is a primary FXR target gene.

Initial characterization of response elements in the first few identified FXR target genes suggested the requirement for an IR-1 element containing two half-sites of the traditional nuclear hormone receptor hexad AGGTCA. This strict definition was challenged by the subsequent finding that a novel ER-8 arrangement in the proximal promoter of the multidrug resistance protein 2 gene (ABCC2) could also function as a potent FXRE (32). Computer-assisted scanning of the sequence flanking the 5' region of the SDC1 gene failed to identify any such traditional FXRE sequences (IR-1 and ER-8) in the 10 kb proximal to the SDC1 transcriptional start site. However, a non-biased method to determine potential FXR/RXR-binding sites (17) suggested that many arrangements of the hexanucleotide repeat can function as binding sites for FXR/RXR heterodimers in vitro.
To identify the cis-acting element responsible for induction of the SDC1 gene in response to FXR, we cloned the 5-kb region flanking the 5’ end of the SDC1 gene into a luciferase reporter construct. Analysis of this construct, as well as constructs with sequential 5’ deletions and mutations, localized the cis-element responsible for induction to a non-consensus DR-1 arrangement at −921 bp relative to the transcriptional start site. Mutation of this element greatly attenuated the FXR-dependent induction of the luciferase reporter gene (Fig. 4). Furthermore, a two copy tandem repeat of the wild-type, but not the mutant DR-1, element highly activated a minimal promoter-reporter construct (Fig. 4). We conclude from these results that the DR-1 element identified here in the SDC1 promoter is a potent FXRE. This hypothesis is supported by competition studies using EMSAs shown in Fig. 5. However, to date, we have been unable to demonstrate direct binding of the FXR/RXR heterodimer to this DR-1 element using EMSAs. It is possible that in vitro a stable complex is not formed because of a high off-rate of FXR/RXR from the DR-1. Alternatively, it is possible that another factor, present in hepatocytes, is required to stabilize the FXR/RXR-DR-1 complex.

The recent identification of four murine and human FXR transcripts and isoforms and the demonstration that the four isoforms are expressed in a tissue-specific manner provide a potential mechanism to control gene expression in different tissues. The finding that both I-BABP (10) and SDC1 are differentially responsive to the four FXR isoforms provides support for this hypothesis. However, whereas I-BABP is poorly activated by FXRα1 and FXRβ1, SDC1 is completely unresponsive to these same two isoforms. Consequently, we conclude that activation of SDC1 by FXR is the first example of an FXR target gene with absolute isoform specificity. Based on these studies, it seems likely that there will be a subset of genes, yet to be identified, that are more responsive to FXRα1 and FXRβ1 (the two isoforms that contain the four-amino acid insert) than FXRα2 and FXRβ2. The identification of such genes would support the concept that FXR isoforms provide an additional level of tissue and target gene specificity.

Over the last few years, identification of FXR target genes has begun to provide insight into the network of physiological functions that are governed by FXR-dependent regulation. As expected from the identification of bile acids as natural FXR ligands, a number of these target genes are involved in cholesterol and bile acid homeostasis (6). A second group of target genes, including PLTP, apoCII, and apoE, are known to be involved in the metabolism of triglyceride-rich plasma lipoproteins. Such processing results in the formation of triglyceride-poor, cholesterol ester-rich lipoprotein remnants that are rapidly removed from the circulation. The current finding that ligand-activated FXR induces SDC1 expression and increases the potential of hepatocytes to participate in lipoprotein uptake provides evidence for a mechanism by which the metabolism and clearance of lipoproteins are coordinately regulated in response to bile acids. The finding 30 years ago that treatment of patients with gallstones with CDCA led to an unexplained decrease in plasma triglycerides (33) is consistent with this hypothesis. Thus, pharmacological activation of FXR may prove to be an alternative treatment for hypertriglyceridemia.

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FIG. 7. Treatment of HepG2-FXR cells with GW4064 leads to increased uptake of 125I-mLDL. A, GW4064 treatment increases surface binding of 125I-mLDL. HepG2-FXR cells were pretreated with 1 μM GW4064 or vehicle for 24 h before exposure to 125I-mLDL (5 μg/ml) and/or LpL (5 μg/ml), as described under “Experimental Procedures.” After 5 h, surface-bound lipoprotein was assessed by treatment with heparin, quantitated, and normalized to total cellular protein. B, GW4064 treatment increases surface binding of 125I-mLDL. Cells were treated as described above, and internalized lipoprotein was assessed as heparin-resistant radioactivity that remained cell-associated. The values were normalized to total cellular protein as described under “Experimental Procedures.” C, GW4064 treatment increases catabolism of 125I-mLDL. Cells were treated as described above, and degraded lipoprotein was quantitated after extraction and normalization to total cellular protein as described under “Experimental Procedures.” The results are representative of three separate experiments.
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On page 20422 under “Results” (lines 18–20), the following sentence was attributed to the incorrect reference: “We chose to explore FXR-mediated regulation of SDC1 based on the fact that it has been reported to be involved in the binding and internalization of plasma lipoproteins (37).” The correct reference is as follows: Fuki, I. V., Kuhn, K. M., Lomazov, I. R., Rothman, V. L., Tuszynski, G. P., Iozzo, R. V., Swenson, T. L., Fisher, E. A., and Williams, K. J. (1997) J. Clin. Investig. 100, 1611–1622.

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