Liver Receptor Homolog 1 Controls the Expression of Carboxyl Ester Lipase*

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The orphan nuclear receptor liver receptor homolog 1 (LRH-1) plays a central role in cholesterol homeostasis by regulating a number of hepatic and intestinal genes critical for reverse cholesterol transport and bile acid homeostasis. Herein, we describe the identification of carboxyl ester lipase (CEL) as a novel target of LRH-1 in pancreas, a tissue in which LRH-1 is abundantly expressed. In situ hybridization and gene expression studies demonstrate that both LRH-1 and CEL are co-expressed and confined to the exocrine pancreas. LRH-1 interacts with a consensus LRH-1 response element in the human CEL promoter, which is perfectly conserved in the rat gene, and induces CEL promoter activity in cotransfection assays. As reported for other LRH-1 target genes, the nuclear receptor short heterodimer partner (SHP) interacts with a consensus LRH-1-induced CEL promoter activity. Chromatin immunoprecipitation demonstrates that binding of LRH-1 to the CEL promoter increases histone H4 acetylation corresponding with the activation of endogenous CEL gene transcription. Our data, identifying CEL as the first pancreatic LRH-1 target gene, indicate that LRH-1 is an important player in enterohepatic cholesterol homeostasis.

Carboxyliester lipase (CEL) (sterol esterase; EC 3.1.1.13), also called bile salt-dependent lipase, bile salt-stimulated lipase, or pancreatic cholesterol esterase, is synthesized within the endoplasmic reticulum of pancreatic acinar cells and secreted into the intestinal lumen as one of the major components of pancreatic juice. CEL binds to the brush border membrane of the small intestine enterocytes in a heparin-dependent fashion (1). Once attached, CEL hydrolyzes dietary cholesteryl esters in the presence of low concentrations of trihydroxylated bile salts. The free cholesterol and fatty acids then released are absorbed by the mucosal cells, where they are re-esterified and transported to the liver as chylomicron particles. CEL has also been shown to be involved in the assembly and secretion of large chylomicrons by the enterocytes of the proximal intestine because it controls the hydrolysis of long chain ceramides (2), known to regulate intracellular lipoprotein trafficking (3, 4). These long chain ceramides are generated from sphingomyelin hydrolysis, which is required for cholesterol uptake by enterocytes. CEL expression is induced at the protein level by cholecystokinin and secretin (5). Activation of Ca2+ and protein kinase C signaling pathways are part of the mechanism by which the acinar cells are induced to secrete CEL (6). In addition, CEL mRNA is induced upon feeding animals a high fat/high cholesterol diet (7).

The liver receptor homolog 1 (LRH-1, NR5A2) is an orphan nuclear receptor that binds as a monomer to the consensus 5′-YCAAGGYCR-3′, the recognition motif for the Ftz-F1 subfamily of nuclear receptors (8–11). LRH-1 is predominantly expressed in tissues of endodermal origin including liver, exocrine pancreas, and intestinal tract (9–12). LRH-1 is involved in the regulation of the expression of transcription factors implicated in early hepatic and pancreatic differentiation such as hepatocyte nuclear factors 3β (12, 13), 4α (13), and 1α (13). It also activates the α-fetoprotein gene (9), which encodes an embryonic marker of liver. Moreover, LRH-1 seems to be involved in the control of cholesterol homeostasis. It has been shown to regulate two of the main enzymes involved in bile acid synthesis from cholesterol, CYP7A1 and CYP8B1 (11, 14–16). CYP7A1 is the rate-limiting enzyme in the classic bile acid synthesis pathway, and CYP8B1 is involved in cholic acid synthesis. The level of CYP8B1 activity determines the hydrophobicity of the circulating bile acid pool, which influences biliary cholesterol and phospholipid secretion as well as intestinal cholesterol absorption. Bile acids inhibit their own synthesis via a farnesoid X receptor-mediated induction of the short heterodimer partner (SHP), an atypical nuclear receptor, which represses LRH-1 activity (15–17). Interestingly, LRH-1 has been shown to be a necessary competence factor for LXR/RXR heterodimer-mediated activation of CYP7A1 (15, 16). LRH-1 is also required for reverse cholesterol transport because it induces the expression of the human cholesteryl ester transfer protein gene (18) and of the scavenger receptor class B type I gene (19), a receptor that mediates selective cellular cholesterol uptake from high density lipoproteins. Finally, in the intestine, it induces the multidrug resistance protein 3, a protein believed to be involved in the transport of bile acids from the enterocyte into blood (20), and mediates the bile acid-dependent negative feedback regulation of the apical sodium-dependent bile acid transporter (21). Together, these findings suggest that LRH-1 is a key player in cholesterol homeostasis.

This study identifies a novel implication for LRH-1 in cholesterol homeostasis through the regulation of CEL transcription. CEL represents the first LRH-1 target gene expressed in the small intestine enterocytes in a heparin-dependent fashion (6). In addition, CEL mRNA is induced upon feeding animals a high fat/high cholesterol diet (7).

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§ The abbreviations used are: CEL, carboxyliester lipase; LRH-1, liver receptor homolog-1; SHP, short heterodimer partner; LXR, liver X receptor; RXR, retinoid X receptor; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation.

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MATERIALS AND METHODS

Recombinant Plasmids—The different CEL promoter luciferase reporter plasmids, CEL1 (−955 to +49), CEL2 (−871 to +49), CEL3 (−667 to +49), and CEL4 (−459 to +49), were generated by cloning different promoter fragments in the XhoI and HindIII sites of the pGL3-basic vector (Promega, Madison, WI). Mutagenesis of the potential LRH-1 binding sites in the CEL3 construct was accomplished with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The forward mutagenic primers (mutated bases underlined) are m1, 5′-AgggAACCTAAACCTTgaggCTAgATCC-3′; m2, 5′-gACAgT-TCCACACCgATgATgATCC-3′; m3, 5′-gCTgTACAgAAATCA-TCCACgATCCACTAgATCC-3′; and m5-1, 5′-AAACgATgATgATCCACTAgATCCACTCCA-3′. Full-length mouse LRH-1 cDNA was subcloned into the expression vector pCMX. The pCMX-LRXα and β expression vectors were a gift from D. J. Mangelsdorf, the CDM8-SHP vector was provided by D. D. Mooney, and the expression vectors were a gift from P. Chambron. All constructs were verified by sequencing. The expression vector CMV-β-gal encoding for β-galactosidase is described elsewhere (22).

Cell Culture and Transient Transfection Assays—CV-1 and HepG2 cell lines were obtained from the European Collection of Cell Cultures (SACEB, Wiltshire, UK), whereas the mouse pancreatic ductal carcinoma LTPA cell line was from ATCC (Manassas, VA). Cells were maintained as specified by the providers. Transfections were carried out by calcium phosphate precipitation (23) or by LipofectAMINE (Invitrogen). Luciferase measurements were normalized to β-galactosidase activity (23).

RNA Analysis and in Situ Hybridization—RNA extraction, Northern blot analysis, and hybridizations were performed as described previously (24). The human CEL cDNA (GenBank accession number M85201, 93 to 892), the full-length human acidic ribosomal phosphoprotein 36B4 (25), the human β-actin (26), and the human LRH-1 cDNA (GenBank accession number U93553) were used as probes. The human multiple tissue Northern blot was from Clontech (Palo Alto, CA). Multiple tissue Northern blot containing human poly(A)+ RNA was hybridized with radiolabeled probes for CEL, LRH-1, or β-actin. A 794-bp fragment (93 to 886) of the human CEL cDNA that recognizes the mouse CEL was cloned between the sites XhoI and HindIII of pBluescript-SK+ (Stratagene) and linearized by NotI. Antisense LRH-1 RNA was synthesized using T7 RNA polymerase (Promega). A 794-bp fragment of the mouse CEL cDNA that recognizes the mouse CEL was cloned between the sites XhoI and HindIII of pBluescript-SK+ and linearized by XhoI. Antisense CEL RNA was synthesized using T7 RNA polymerase (Promega).

EMSAs and Oligonucleotide Sequences—Mouse LRH-1 was synthesized in vitro using the TNT T7-quick coupled rabbit reticulocyte lysate system (Promega) and the expression vector pCMX-LRHX-1. Molecular weight and quality of the proteins were verified by SDS-PAGE. Electrophoretic mobility shift assays (EMSA) were performed as described previously (23). For supershift experiments, an anti-LRH-1 antibody (kroyal) provided by C. Dreyer (28) was allowed to interact in the binding buffer with the in vitro synthesized LRH-1. After 15 min, radioactive probe was added. The total mixture was incubated for 20 min and loaded on gel. For competition experiments, increasing amounts (from 10- to 200-fold molar excess) of unlabelled oligonucleotide (5′-gATCCgATgATgATgACgCTCCA-3′, m5-2, 5′-gATCCgATgATgATgACgCTCCA-3′, and m5-3, 5′-gATCCgATgATgATgACgCTCCA-3′) (mutated bases underlined) were included in the reactions.

ChIP Assays—For chromatin immunoprecipitation, an anti-acetylated 3H histone antibody (Euromedex, Mundolsheim, France) and an anti-LRHX1 antibody (28) were used. Cellular proteins were cross-linked to DNA with 1% formaldehyde at 21 °C for 10 min. After cell lysis, three 9-s cycles of sonication were performed to prepare DNA fragments ranging in size from 200 to 1000 bp. Next, lysates were precleared by incubation with protein A-Sepharose. Immunoprecipitation was then carried out overnight at 4 °C using dilutions of 1/400 for the anti-acetyl H4 histone and of 1/80 for the anti-LRX-1 antibody. The antibody-DNA complex was precipitated using protein A-Sepharose. After washing and elution, the cross-linking was reversed by heating the samples at 65 °C overnight. The DNA was then purified and used for PCR amplification using quantitative PCR.

Quantitative PCR—Quantification was carried out by real-time PCR using a LightCycler and the DNA double-strand specific SYBR Green I dye for detection (Roche, Basel, Switzerland). The primer pairs used to amplify mouse LRH-1, mouse/human 36B4, and human CEL cDNAs were: 5′-CTTTgAgcTATCTgAgATTTTCACC3′ and 5′-ATACAgTAAT-TCACATTATTTAAAT3′, 5′-ATgCTgAAgTACATgTCACCAC3′ and 5′-gTg- TAATCCgCTTCCACCgACTACAA3′, and 5′-AggAggTgAgATCTgAACgATgAAgAC3′ and 5′-gAAAggTgACCACgATgACgT3′, respectively. The primer pairs used to amplify a CEL promoter domain containing s5 and to amplify a region that does not contain s5 were: 5′-ACCAAgCCTgTgAA-CAAA3′ and 5′-TCTgATTgAgcAgAgCA3′, and 5′-CTTgAgCT-CAGAgTgAgATCTgAgAC3′ and 5′-gTgAgAgTgAgCatgtgACCgACCA3′, respectively.

FIG. 1. CEL and LRH-1 mRNA are co-expressed in the pancreas. A, Northern blot containing human poly(A)+ RNA was hybridized with radiolabeled probes for CEL, LRH-1, or β-actin. Filled arrowhead, transcripts; open arrowhead, different sizes of the muscle-specific β-actin transcripts. B, blots of total RNA from the indicated mouse tissues (top) were hybridized with radiolabeled probes for CEL and 36B4. Reverse transcription-PCR on RNA from the same mouse tissues was performed to amplify LRH-1 (285 bp) and 36B4 (420 bp) mRNAs (bottom). The arrowheads indicate the mRNAs and the specific amplification products. C, in situ hybridization was performed on adult mouse pancreas using 35S-labeled antisense RNA probes. Toluidine blue staining is depicted on the left. Results of the in situ hybridization with 35S-labeled antisense RNA probes are represented on the right, and the inset shows the results of the hybridization with the corresponding 35S-labeled sense RNA probes, used as a negative control. Islets, identified by immunofluorescence against insulin (data not shown), are outlined with a white contour.

antibody-DNA complex was precipitated using protein A-Sepharose. After washing and elution, the cross-linking was reversed by heating the samples at 65 °C overnight. The DNA was then purified and used for PCR amplification using quantitative PCR.

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RESULTS

Expression of CEL Co-localizes with LRH-1 Expression in the Exocrine Pancreas—Expression of the genes encoding for LRH-1 and CEL was determined in a number of human and murine tissues by Northern blot hybridization and reverse transcription-PCR (Fig. 1). In humans and mice, CEL mRNA was detected only in the pancreas, a tissue also particularly enriched in LRH-1 (Fig. 1, A and B). Co-expression of CEL and LRH-1 in the pancreas was confirmed at the subcellular level by in situ hybridization on sections of mouse pancreas, where the expression of CEL and LRH-1 was confined to the exocrine structures (Fig. 1C). As reported previously (9–12), LRH-1 is also expressed in mouse intestine (Fig. 1B, lane 2), as well as in murine and human liver (Fig. 1, B, lane 3, and A, lane 5, respectively). The observation that CEL and LRH-1 are co-expressed in the exocrine pancreas pointed to the possibility that LRH-1 might control the expression of CEL in this tissue.

The CEL Promoter Is Activated by LRH-1—To determine whether LRH-1 regulates transcription of the CEL gene, CV-1 cells were cotransfected with a human CEL promoter-luciferase reporter construct, containing sequences between 955 bp and +49 (CEL1) (Fig. 2A), and an LRH-1 expression vector.
A 6-fold increase in CEL promoter activity was observed (Fig. 2B). In addition, a 3.9-fold induction of the CEL promoter activity was seen upon LRH-1 cotransfection of the mouse pancreatic LTPA cell line (Fig. 2C). These observations suggest that LRH-1 activates the CEL promoter.

To localize the potential response element(s) in the CEL promoter mediating LRH-1 regulation, CEL1, CEL2, CEL3, and CEL4 deletion constructs (Fig. 2A) were generated and assayed for responsiveness to LRH-1 in CV-1 cells. Cotransfection of LRH-1 induced the CEL1, CEL2, and CEL3 reporter constructs to a similar extent, whereas a drop in promoter activation was observed with the CEL4 construct (Fig. 2B). Because construct CEL3 retained most of the promoter activity in response to LRH-1 cotransfection, we focused on the sequence between -667 and +49 to further map the regulatory element(s) mediating the effect of LRH-1.

LRH-1 Stimulates CEL Gene Transcription through Its Direct Interaction with s5—Five sequence motifs, designated s1 to s5, with homology to the consensus Ftz-F1 motif 5'-YCAAG-GYCR-3', were identified in the region spanning the nucleotide -667 up to +49 of the human CEL promoter (CEL3) (Fig. 3A). One of these, s5, is perfectly conserved between the rat (29) and human genes (Fig. 3B) and is highly homologous to LRH-1 response elements previously identified in the promoter of different LRH-1 target genes (Fig. 3C). EMSA were used to investigate binding of in vitro synthesized LRH-1 to these potential response elements in the CEL promoter (s1 to s5; Fig. 3A).

An intense shifted band was observed only with the s5 probe (Fig. 4A). This band was no longer present upon addition of an anti-LRH-1 antibody that interferes with LRH-1 DNA binding domain, showing the specific binding by LRH-1. CEL s5 and an LRH-1 binding site (TCAAGGTTG) in the SHP promoter (30) were capable of binding LRH-1 with comparable affinity (data not shown). When increasing concentrations of unlabeled, wild-type oligonucleotide representing s5 were added as competitor, binding of LRH-1 to the CEL s5 probe was inhibited dose-dependently (Fig. 4B, lanes 3–5). Addition of up to 200-fold molar excess of two different mutated oligonucleotides corresponding...
sequences were unable to bind LRH-1 in EMSA (Fig. 4A). Likewise, the two mutant s5 segments showed that LRH-1 actually binds and transactivates the CEL promoter in vivo. To unequivocally demonstrate which of the five putative sequence elements in the CEL promoter confers responsiveness to LRH-1, all five potential sites (Fig. 3A) were mutated individually in the CEL3 reporter construct, and their activity was then compared in CV-1 cells. Although mutation of the s5 element (m5-1) resulted in a complete loss of activation of the CEL promoter by LRH-1, mutations in s1 (m1), s2 (m2), s3 (m3), and s4 (m4), retained complete ability to respond to LRH-1 (Fig. 4C). These results are consistent with those of the EMSA and underscore the idea that s5, the only LRH-1 response element completely conserved between rat and human, is the principal mediator for the LRH-1 response.

LRH-1 Binds and Activates the CEL Promoter in Vivo—To show that LRH-1 actually binds and transactivates the CEL promoter in vivo, chromatin immunoprecipitation (ChIP) studies were performed using antibodies against LRH-1 and acetylated H4 histones. Acetylation of histones has been shown to stimulate transcription initiation by facilitating access of transcription factors. HepG2 cells were transfected with either an empty expression vector or an LRH-1 expression vector. Upon transfection with LRH-1, specific binding of LRH-1 to the CEL promoter was observed as evidenced by the presence of the band corresponding to the amplification product of the CEL promoter region containing the s5 site (Fig. 5A, top, compare lanes 1 and 2). In contrast, the anti-LRH-1 antibody did not immunoprecipitate a region of the CEL promoter distant from s5 (Fig. 5A, center). Occupancy of the CEL promoter by LRH-1 was also associated with an increase in H4 histone acetylation (Fig. 5A, bottom), which corresponds to a 3.8-fold induction as quantified by real-time PCR (Fig. 5B). These observations in the ChIP assay were further corroborated by a 3.7-fold induction of endogenous CEL mRNA levels in response to LRH-1 overexpression (Fig. 5C). In combination, these results establish that LRH-1 binds in vivo to the human CEL promoter and induces an increase in H4 histone acetylation, facilitating subsequent transcriptional initiation.

The CEL Promoter Is Regulated by LRH-1 and SHP, but Not by LXR/RXR Heterodimers—SHP was previously shown to inhibit LRH-1 activity on a number of LRH-1 target promoters (15–19, 21). To test whether SHP also inhibits the LRH-1-mediated induction of CEL promoter activity, increasing amounts of a SHP expression vector were cotransfected with a fixed amount of LRH-1 and the CEL3 reporter construct. Significant attenuation of the activation of the CEL promoter by
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Fig. 6. The CEL promoter is regulated by LRH-1 and SHP, but not by LXR. A, increasing concentrations of SHP expression vector (respective ratios of SHP:LRH-1 are 0:1 (lane 3), 1/10:1 (lane 4), and 1:1 (lane 5)) decrease dose-dependently LRH-1-induced CEL3 promoter activity in transient cotransfections of CV-1 cells. B, the CEL1 promoter was cotransfected in CV-1 cells with expression vectors for RXRα and LXRα (lanes 5–8) in the absence (open bars) or presence (gray bars) of the rexinoid LG 100268 (10–6 m; 268) and the LXR agonist 22(R)-OH-cholesterol (10–5 m; 22R) and cotransfected LRH-1 (lanes 2, 7, and 8).

LRH-1 was observed upon cotransfection of SHP (Fig. 6A, compare lanes 3 and 4–5).

In view of the reported synergism between LRH-1 and LXR (15, 18), we tested whether this observation could also be further generalized to the CEL promoter. Interestingly, cotransfection with LXRun/RXR or LXRβ/RXR did not affect the activity of the CEL reporter construct CEL1 in the presence or absence of their cognate ligands, 22-(R)-OH-cholesterol and LG 100268 (Fig. 6B and data not shown).

DISCUSSION

In this study, we provide evidence that CEL expression is subject to regulation by LRH-1. CEL and LRH-1 mRNAs are co-expressed in the exocrine pancreas, compatible with a regulatory role of LRH-1 on CEL expression. LRH-1 induces hCEL promoter activity through a direct interaction with the consensus s5 LRH-1 response element, which was identified by a combination of transient transfection and EMSA experiments. None of the other potential LRH-1 binding sites upstream of s5 interacted with LRH-1. In vivo evidence for the direct regulation of the CEL promoter by LRH-1 was furthermore obtained by chromatin immunoprecipitation experiments, which demonstrated in vivo binding of LRH-1 to the promoter. Moreover, ChIP assays also showed an increase in H4 histone acetylation on the CEL promoter, indicating that the occupancy of this promoter by LRH-1 is associated with a transcriptional active status. These findings are further corroborated by the observed increase of endogenous CEL mRNA levels in cells that overexpress LRH-1.

Interestingly, LRH-1 responsiveness was reduced for the smallest reporter construct CEL4 that still harbors the s5 site. This partial reduction in LRH-1 responsiveness could point to the existence of additional factor(s) acting upstream of s5 that contribute to LRH-1-mediated activation of the CEL promoter. Indeed, LRH-1 and steriodogenic factor-1, another receptor of the Ptf/P1 family, are known to act in synergism with other transcription factors. A transcriptional cooperation between LRH-1 and LXR (15, 18) and between steriodogenic factor-1 and other transcription factors such as Egr-1 and CREB (31, 32) has been reported on other target genes. Most often, such a cooperation translates in a synergistic transcriptional activation. We excluded a cooperative effect between LRH-1 and LXR on CEL promoter activity (see below). We therefore speculate that the reduced activity of the CEL4 reporter relative to longer CEL reporters is caused by the loss of cooperation with another transcription factor, also unidentified, binding to the CEL promoter.

High fat/high cholesterol feeding has been shown to induce CEL mRNA levels (7). In view of the activation of the LXR/RXR signaling pathway by cholesterol (33) and the reported synergism between LRH-1 and LXR (15, 18), we tested whether LXRun/RXR could activate the CEL promoter. Cotransfection with LXRun/RXR or LXRβ/RXR, in the presence of their cognate ligands, did not affect the activity of the CEL promoter, an effect that was not changed by the presence of LRH-1. The absence of a regulatory effect of LXR on CEL expression is furthermore bolstered by the absence of LXR expression in the exocrine pancreas (M. Dubois, K. Schoonjans, J. Auwerx, unpublished data). Hence, another factor might be involved in the high fat/high cholesterol induction of CEL mRNA levels.

SHP has been reported to interact with a variety of nuclear receptors (34–41). In most cases, SHP initially inhibits indirectly the activity of its interacting partners by coactivator competition (39, 42). Once bound to the receptor, SHP can act as a direct transcriptional repressor (17, 36, 39). SHP was previously shown to inhibit the LRH-1-mediated activation of the CYP7A1, human cholesteryl ester transfer protein, apical sodium-dependent bile acid transporter, and scavenger receptor class B type I promoters (15–19, 21). In line with these data, we demonstrate here that SHP also attenuates the LRH-1-mediated induction of CEL promoter activity.

CEL is a key gatekeeper of dietary cholesterol absorption, as it catalyzes the hydrolysis of cholesteryl esters in the intestinal lumen. Dietary cholesterol becomes incorporated through the action of bile acids into mixed micelles and is absorbed as free cholesterol by intestinal cells. Cholesteryl esters require hydrolysis before their absorption. Compared with control mice, CEL null mice absorb only half the amount of cholesteryl esters, confirming a primary role for CEL in hydrolysis and, consequently, absorption of cholesterol from cholesteryl esters (43, 44). In addition, CEL has a wide substrate specificity in that it has been shown to hydrolyze not only cholesteryl esters but also triglycerides, phospholipids, lysophospholipids (reviewed in Ref. 45), vitamin esters (46), retinyl esters (47), and lipomides (48), suggesting an important role for this enzyme in the digestion and absorption of a broad spectrum of dietary lipids. In view of its enterohepatic expression and its regulatory role in reverse cholesterol transport and bile acid homeostasis, through its effect on human cholesteryl ester transfer protein,
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scavenger receptor class B type I, CYP7A1, CYP8B1, apical sodium-dependent bile acid transporter, and multidrug resistance protein 3 expression, LRH-1 has been proposed as a key factor in cholesterolemia. The current study, identifying CEL as the first LRH-1 target gene expressed in the exocrine pancreas, lends further support to this hypothesis. The role of CEL in the hydrolysis of dietary lipids and chylomicron assembly and secretion might, however, point to a more complex role of LRH-1 that goes beyond the regulation of reverse cholesterol transport and bile acid homeostasis, and suggests that LRH-1 could control other critical steps in cholesterol homeostasis.

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