Liver receptor homolog 1 controls the expression of the scavenger receptor class B type I

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The scavenger receptor class B type I (SR-BI), which mediates selective cellular cholesterol uptake from high-density lipoproteins (HDLs), plays a key role in reverse cholesterol transport. The orphan nuclear receptor liver receptor homolog 1 (LRH-1) and SR-BI are co-expressed in liver and ovary, suggesting that LRH-1 might control the expression of SR-BI in these tissues. LRH-1 induces human and mouse SR-BI promoter activity by binding to an LRH-1 response element in the promoter. Retroviral expression of LRH-1 robustly induces SR-BI, an effect associated with histone H3 acetylation on the SR-BI promoter. The decrease in SR-BI mRNA levels in livers of LRH-1^{-/-} animals provides *in vivo* evidence that LRH-1 regulates SR-BI expression. Our data demonstrate that SR-BI is an LRH-1 target gene and underscore the pivotal role of LRH-1 in reverse cholesterol transport.

INTRODUCTION

The transport of cholesterol from peripheral cells, including those in the arterial wall, to the liver for excretion is termed reverse cholesterol transport (RCT). High-density lipoproteins (HDLs) are the principle vehicles for RCT, and their concentration is inversely correlated to the incidence of coronary artery disease (reviewed in Krieger, 1999). In addition, HDLs also deliver cholesterol to steroidogenic tissues for hormone synthesis. The scavenger receptor class B type I (SR-BI) is a cell surface HDL receptor that mediates selective cholesterol uptake from HDLs (reviewed in Krieger, 1999). SR-BI is expressed at high levels in both liver and steroidogenic tissues and is a key regulator of HDL cholesterol flux. As a consequence, hepatic overexpression of SR-BI reduces plasma HDL levels (Wang *et al.*, 1998; Ueda *et al.*, 1999; Kozarsky *et al.*, 2000), concomitant with an

increase in cholesterol excretion (Kozarsky *et al.*, 1997; Sehayek *et al.*, 1998). Conversely, SR-BI knock-out (KO) mice have increased plasma cholesterol levels and are predisposed to atherosclerosis, leading to spontaneous myocardial infarction, when crossed with apo E KO mice (Trigatti *et al.*, 1999; Braun *et al.*, 2002).

Liver receptor homolog 1 (LRH-1, NR5A2) is a nuclear orphan receptor that binds as a monomer to the DNA sequence 5'-YCA AGG YCR-3', the recognition motif for the fushi tarazu factor 1 (Ftz-F1) subfamily of nuclear receptors, and is predominantly expressed in tissues from endodermal origin. LRH-1 exercises a critical role during development by controlling the expression of the α -fetoprotein (Galarneau *et al.*, 1996) and a number of transcription factors critical for hepatic and pancreatic development, i.e. hepatic nuclear factors HNF-3 β , HNF-4 α and HNF-1 α (Paré *et al.*, 2001). In the adult, the physiological role of LRH-1 has been linked to the control of cholesterol and bile acid homeostasis. Two of the main enzymes involved in bile acid synthesis are regulated by LRH-1: CYP7A1 (Nitta *et al.*, 1999), the rate-limiting enzyme for bile acid synthesis; and CYP8B1 (del Castillo-Olivares and Gil, 2000), whose activity determines the ratio of cholic to chenodeoxycholic acid. LRH-1 has also been reported to regulate the expression of cholesterol ester transfer protein (CETP; Luo *et al.*, 2001), which plays a predominant role both in the remodeling of HDL particles and in RCT. For a number of target genes, LRH-1 together with the liver X receptor (LXR), the farnesol X receptor (FXR) and the co-repressor short heterodimer partner (SHP), are part of a concerted nuclear receptor network that ensures the adequate gene expression in response to intracellular cholesterol and bile acid levels (Goodwin *et al.*, 2000; Lu *et al.*, 2000; del Castillo-Olivares and Gil, 2001; Luo *et al.*, 2001).

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Fig. 1. SR-BI and LRH-1 mRNA are co-expressed in the liver and ovary but not in testis and adrenal. Toluidene Blue staining (first column) and *in situ* hybridization of tissue sections hybridized with antisense (second column) and sense (third column) RNA probes for LRH-1 and SR-BI. **(A)** Co-expression of SR-BI and LRH-1 in parenchymal liver cells and gall bladder epithelium. lc, liver capsule/subserosa of gallbladder; gb, gall bladder mucosa. **(B)** Adrenal cortex expresses SR-BI, with highest levels in the zona fasciculata and zona reticularis, but does not express LRH-1. **(C)** LRH-1 and SR-BI are both expressed in the corpus luteum (cl) of the ovary. LRH-1 is highly expressed in the granulosa cell layer (gr) of Graafian (gf) and preantral (pf) follicles, with only moderate levels of SR-BI. SR-BI, but not LRH-1, is expressed in the theca layer (th) and interstitial tissue (is). cr, corona radiata. **(D)** Section of testis expressing SR-BI but not LRH-1.

Here, we show that LRH-1 and SR-BI are co-expressed in liver and ovary and demonstrate that LRH-1 governs the expression of the SR-BI gene both *in vitro* and *in vivo*. These data emphasize the physiological importance of LRH-1 in a broader context than originally proposed and add further evidence to a role for LRH-1 in the control of cholesterol transport.

RESULTS

SR-BI and LRH-1 expression co-localize in liver and ovary

In the liver, cholesterol is used for bile acid synthesis, secretion into bile or assembly and re-export in the form of very-low-density lipoproteins (VLDLs). In steroidogenic tissues, such as adrenal cortex, testis and ovary, cholesterol is either stored or used for hormone synthesis. SR-BI is expressed at high levels in both of these cholesterol-metabolizing tissues. The expression of LRH-1 mRNA was therefore analyzed in these tissues by *in situ* hybridization and compared to the expression profile of SR-BI in adult mice. LRH-1 was expressed in liver but, in contrast to SR-BI, not in adrenal and testis (Figure 1A, B and D). In the ovary, LRH-1 was abundantly expressed in the granulosa cells of follicles at different stages of development, including preantral and mature Graafian follicles, and in the corpus luteum. No LRH-1 was detected in interstitial tissue and in the theca interna

and externa (Figure 1C). SR-BI is, however, present in these structures (Figure 1C). The co-expression of SR-BI and LRH-1 in liver and certain ovarian structures indicates that LRH-1 might control the expression of SR-BI in these tissues.

The SR-BI promoter is activated by LRH-1

To determine whether LRH-1 regulates the transcription of SR-BI, transfection assays were performed. Co-transfection in CV1 cells of a luciferase reporter containing ± 3 kb of the promoter of the human SR-BI (CLA-I) gene and an expression vector for LRH-1 resulted in a 5-fold increase in reporter activity (Figure 2A). A reporter driven by > 2 kb of the mouse SR-BI promoter was also responsive to LRH-1, although to a weaker extent (2-fold) (Figure 2A).

Since LRH-1 has been reported to act as a competence factor for LXR (Lu *et al.*, 2000; Luo *et al.*, 2001), we examined whether the concerted expression of LRH-1, LXR and the retinoid X receptor (RXR) in the presence of the synthetic LXR ligand T0901317 affected SR-BI promoter activity. The heterodimer LXR/RXR did not influence the activity of the SR-BI reporter (Figure 2B), and adding LRH-1 did not result in a synergism as observed for the CYP7A1 gene (Lu *et al.*, 2000). SHP was previously shown to inhibit LRH-1 activity on a number of genes (Goodwin *et al.*, 2000; Lu *et al.*, 2000; del Castillo-Olivares and

Fig. 2. Activity of the SR-BI promoter is induced by LRH-1. (A) The activity of the mSR-BI and hSR-BI reporter constructs in CV1 cells transfected either with an empty vector (pCMX) or an mLRH-1 expression vector (LRH-1). (B) The hSR-BI promoter was co-transfected in CV1 cells with expression vectors for LRH-1, RXR α and LXR α in the presence or absence of the LXR agonist T0901317 (10 μ M). (C) Increasing amounts of SHP (ratios of LRH-1/SHP are 1/0.1, 1/0.5 and 1/1) decrease LRH-1-induced SR-BI promoter activity in CV1 cells. (D) Illustration of the nested deletion constructs SR-BI1 (-2913), SR-BI2 (-258), and SR-BI3 (-58) of the hSR-BI promoter. The shaded box represents the putative LRH-1 response element. (E) Effect of transfection with empty or LRH-1 expression vector on the activity of the hSR-BI reporter deletions.

Gil, 2001; Luo *et al.*, 2001). Consistent with this, SHP attenuated dose-dependently the LRH-1-mediated induction of the SR-BI promoter (Figure 2C).

To localize more precisely the LRH-1 response element(s) in the hSR-BI promoter, the deletion constructs SR-BI1 (-2913 to +135 bp), SR-BI2 (-258 to +135) and SR-BI3 (-58 to +135) (Figure 2D) were assayed for responsiveness to LRH-1 in CV1 cells. LRH-1 induced the SR-BI1 and SR-BI2 constructs to a roughly similar extent (5- and 3-fold, respectively), whereas a brisk drop in promoter activation was observed with the SR-BI3 construct (Figure 2E). Since the SR-BI2 construct retained the majority of response to LRH-1, we concentrated on the sequence between -258 and -58 to further map the potential LRH-1 response element (RE).

LRH-1 stimulates SR-BI expression through a unique LRH-1 RE

The fragment between -258 and -58 of the hSR-BI promoter contained one sequence element with strong homology to the consensus Ftz-F1 motif (between -77 and -69; Figure 3A). Using this element in electrophoretic mobility shift assays (EMSAs), a

retarded protein complex was detected in the presence of LRH-1 (Figure 3B, lane 1). This shifted band was dose-dependently decreased by unlabeled wild type (Figure 3B, lane 2–4) but not by a mutant oligonucleotide (Figure 3B, lane 5). The mutated SR-BI LRH-1 RE was unable to bind LRH-1 (Figure 3B, lane 6).

The LRH-1 RE was next modified by *in vitro* mutagenesis in the context of the hSR-BI1 and hSR-BI2 reporter constructs (Figure 3C), and their response to LRH-1 was compared. Mutation of this LRH-1 RE attenuated the activity of the reporter constructs in response to LRH-1 (Figure 3C), demonstrating that it is the principal site transmitting the effect of LRH-1 on the SR-BI promoter.

LRH-1 binds and activates the SR-BI promoter *in vivo*

We next addressed whether LRH-1 could regulate the expression of the endogenous SR-BI gene. Therefore, cells of hepatic and pancreatic origin, i.e. BNL CL.2 and LTPA cells, were transduced with a retrovirus encoding mouse and human LRH-1 or a control retrovirus. Only cells infected with retroviruses encoding LRH-1, but not with the empty virus, expressed LRH-1

Fig. 3. Identification of LRH-1 binding site in the hSR-BI promoter. **(A)** Binding site for LRH-1 identified in the SR-BI1 promoter construct (SR-BI_{wt}). Consensus LRH-1 RE (Consensus) and the mutated construct (SR-BI_{mt}) are also shown. **(B)** LRH-1 binds to the SR-BI_{wt} element in EMSAs (lane 1). Binding of LRH-1 to the radiolabeled SR-BI_{wt} oligonucleotide was dose-dependently decreased upon addition of 10-, 100- or 200-fold molar excess of cold oligonucleotide (comp). Only the double-stranded oligonucleotide SR-BI_{wt}, but not SR-BI_{mt} (200-fold molar excess, lane 5), is able to compete for binding of LRH-1. LRH-1 does not bind to the SR-BI_{mt} oligonucleotide (lane 6). **(C)** The SR-BI1 and SR-BI2 reporter constructs carrying either the wild-type (wt) or mutated (mt) LRH-1 binding site were co-transfected in CV1 cells with an empty pCMX vector or an LRH-1 expression vector.

(Figure 4A). Interestingly, the expression of SHP, a known target gene of LRH-1 (Lee *et al.*, 1999), and SR-BI were both similarly induced in cells that express LRH-1.

To prove that LRH-1 binds and transactivates the SR-BI promoter *in vivo*, chromatin immunoprecipitation (ChIP) assays were initially performed in cells that express LRH-1 endogenously. To this end, an antibody specific to acetylated histone H3 was used to immunoprecipitate the chromatin from rat McA-RH7777 hepatoma cells. A strong H3 hyperacetylation was observed on the 5' regulatory region of the SR-BI gene, but not on regions downstream of the LRH-1 RE (Figure 4B). To further investigate whether this hyperacetylation was due to the presence of LRH-1, histone H3 acetylation levels were analyzed in mouse LTPA cells infected with a mock or an LRH-1 expressing retroviral vector (Figure 4C). Only infection with the mLRH-1 retrovirus elicited a robust H3 histone hyperacetylation of the chromatin packing the SR-BI promoter. Examination of downstream regions residing outside the RE (SR-BI ex) and the promoter of the β -actin housekeeping gene revealed no significant differences in histone H3 acetylation between both LTPA cell lines (Figure 4C). Taken together, these data indicate that the effect on H3 acetylation is specific and most likely secondary to the presence of LRH-1 on the promoter.

Finally, to demonstrate the *in vivo* relevance of the regulation of SR-BI by LRH-1, SR-BI expression was analyzed in heterozygous LRH-1 KO mice. These LRH-1^{+/-} mice were generated from targeted 129Sv ES cells by Cre recombinase-mediated

excision of LoxP flanked exons 3 and 4. A detailed description of these mice will be the subject of a separate manuscript (J.-S. Annicotte and J. Auwerx, in preparation). LRH-1 levels were significantly reduced in the livers of these LRH-1^{+/-} mice. Consistent with the regulation of SR-BI expression by LRH-1, livers of the LRH-1^{+/-} mice expressed significantly lower levels of SR-BI mRNA (Figure 5). Expression of the established LRH-1 target gene SHP (Lee *et al.*, 1999) was also significantly reduced in the livers of heterozygous LRH-1 KO mice, whereas no difference could be observed for RXR α and LXR α .

DISCUSSION

SR-BI plays an important role in the selective uptake of cholesterol by the liver and steroidogenic organs. Both LRH-1 and SR-BI are expressed in liver and ovary but not in testis and adrenals, which only express SR-BI. Testis and adrenal, however, express the steroidogenic factor 1 (SF-1), another receptor of the Ftz-F1 subfamily of nuclear receptors, which recognizes a similar RE as LRH-1 (Ikeda *et al.*, 1993). Interestingly, SF-1 was previously shown to bind and activate the hSR-BI promoter (Cao *et al.*, 1997). Our findings demonstrating a co-expression of SR-BI and LRH-1 in liver and ovary prompted us to characterize the regulation of SR-BI expression by LRH-1. In this study, we provide conclusive evidence that LRH-1 induces the activity of the hSR-BI and mSR-BI promoters *in vitro*. The significance of this regulation was highlighted *in vivo* by ChIP experiments, which

Fig. 4. SR-BI is regulated by LRH-1 *in vivo*. (A) SR-BI mRNA is induced both in hepatic BNL CL.2 and in pancreatic LTPA cells infected with a retrovirus encoding either mLRH-1 or hLRH-1 but not in cells infected with a control retrovirus (–). (B) ChIP was performed using a preimmune serum (PI) or an antibody recognizing acetylated histones H3 (H3) in rat McA-RH7777 cells expressing LRH-1 endogenously. The input and immunoprecipitated (IP) DNA was assayed by PCR to amplify the SR-BI promoter region containing the LRH-1 RE (SR-BI 5'), exon 2 of the mSR-BI gene (internal control, SR-BI ex) and a β -actin housekeeper gene promoter fragment (β -actin 5') that served as an external control. (C) ChIP was performed in mouse LTPA cells infected with a mock (–) or a mLRH-1 (LRH-1) retrovirus, as described in (B). Primers used to amplify the mouse promoter (SR-BI 5'), the internal control (mouse exon 11, SR-BI ex) and the external control (β -actin 5') are described in Methods.

Fig. 5. Expression of SR-BI mRNA is reduced in livers of LRH-1^{-/-} mice. Northern blot hybridizations were performed with cDNAs for LRH-1, SR-BI, SHP, LXR α , RXR α and 36B4.

demonstrated an increase in histone acetylation upon the binding of LRH-1 to the SR-BI promoter. The physiological relevance of SR-BI regulation by LRH-1 was underscored by the induction of SR-BI mRNA in two distinct cell lines that express either mLRH-1 or hLRH-1 and by the reduction in SR-BI mRNA levels in the livers of heterozygous LRH-1 KO mice.

Genes involved in metabolic control are often co-regulated by multiple nuclear receptors. SR-BI forms no exception to this paradigm. In fact, SHP attenuates LRH-1-induced SR-BI promoter activity, an interaction that is of physiological relevance, since both proteins are co-expressed in the liver and a number of other tissues. LRH-1 has been demonstrated to act as a competence factor for LXR to synergistically stimulate certain pathways of cholesterol homeostasis, such as those affected by CYP7A1 and CETP. On other genes, LRH-1 and LXR act

independently. For example, inhibition of cholesterol uptake by the stimulation of ABCA1 in the intestine is clearly dependent on LXR (Repa *et al.*, 2000), whereas it seems not to be affected by LRH-1 (Luo *et al.*, 2001). Conversely, our data suggest that SR-BI expression is controlled by LRH-1 but not by LXR.

It is becoming clear that LRH-1 not only controls cholesterol excretion by stimulating the synthesis of bile acids, but also functions in total body cholesterol homeostasis in general and in RCT in particular. This theory is underpinned by the enrichment of LRH-1 expression in tissues that metabolize cholesterol and also because LRH-1 has been reported to regulate CETP, which plays a predominant role in both the remodeling of HDL particles and in RCT. The current study, identifying SR-BI as an LRH-1 target in liver and ovary, adds further evidence to this hypothesis.

METHODS

Materials. T0901317 was a gift of J. Lehmann (Strasbourg, France). The anti-acetyl H3 histone antibody was purchased from Euromedex, Souffelweyersheim, France.

Recombinant plasmids. The hSR-BI and mSR-BI promoter luciferase reporter plasmids were generated by cloning fragments corresponding to –2913 to +135 and –2015 to +102 of the hSR-BI and mSR-BI genes into the pGL3 basic vector (Promega, Madison, WI). The hSR-BI2 (–258 to +135) and hSR-BI3 (–58 to +135) 5' deletion constructs were generated from hSR-BI by *Stu*I and *Rsa*I restriction digests, respectively. Mutagenesis of the LRH-1 binding site was accomplished by the Gene Editor protocol (Promega) using the mutagenic primer 5'-CCTGAAGCCCACTTCTGCCCCGGGGGC-3'. mLRH-1 and

K. Schoonjans *et al.*

hLRH-1a were subcloned in the retroviral vector pLPCX (Clontech). Full-length mLRH-1 cDNA was cloned into the expression vector pCMX. pSG5-RXR α , pCMX-LXR α and β and CDM8-SHP were respective gifts of P. Chambon (IGBMC, Illkirch, France), D. Mangelsdorf (UTSWMC, Dallas, TX) and D. Moore (Baylor College, Houston, TX).

Cell culture, transient transfection assays and retroviral infection. Mouse (BNL CL.2 and LTPA) and rat (McA-RH7777) cells were maintained and grown as specified by the American Type Culture Collection. Transfections were carried out by calcium phosphate precipitation (Schoonjans *et al.*, 1996) or by lipofectamine (Life Technologies, Burlington, Canada). Luciferase values were normalized to an internal β -galactosidase control. Transfection data represent the mean ($\pm$ standard deviation) of triplicate experiments. Retrovirus production and infection of BNL CL.2 and LTPA cells was performed exactly as described previously (Brendel *et al.*, 2002).

Animals. Ten-week-old C57Bl/6 mice were used for *in situ* hybridization. LRH-1^{+/−} mutant mice were generated from targeted 129Sv ES cells by Cre recombinase-mediated excision of exons 3 and 4, which were flanked by LoxP sites.

RNA analysis. RNA extraction and northern blot analysis were performed as described previously (Schoonjans *et al.*, 1996). ³²P-labeled cDNAs corresponding to mSR-BI (DDBJ/EMBL/GenBank accession number U37799), hLRH-1 (U93553), mLRH-1 (M81385), mSHP (L76567), mLXR α (NM_013839), mRXR α (NM_011305) and the human acidic ribosomal phosphoprotein 36B4 (loading control) were used as probes.

EMSAs. EMSAs using *in vitro*-synthesized hLRH-1 (TNT T7, Promega) were performed as described previously (Schoonjans *et al.*, 1996). For competition experiments, increasing amounts of cold oligonucleotide (wild type, 5'-CTGAAGCCCAAGGCT-GCCCGG-3'; mutant, 5'-CTGAAGCCCACTTCTGCCCGG-3') (mutated bases underlined) were used.

ChIP assays. ChIP assays were essentially performed as described previously (Takahashi *et al.*, 2000). Input and immunoprecipitated DNA were purified and used to amplify promoter fragments of mSR-BI (546 bp; 5'-TTCCGAATGGGGAAGT-GCAAAG-3' and 5'-GTCCGCGTGCGCGGAAGGGGCTCT-3'), rSR-BI (506 bp; 5'-GCGACTGCAATTGATGCAGGG-3' and 5'-GTCCGCGTGCGCGCCGAGAGTCCG-3') and m/r β -actin (200 bp; 5'-GCGGCCAACGCCAACTCTCC-3' and 5'-GGC-CCCGCGCCGCTCACTCAC-3'). Amplification of internal control fragments residing outside the RE region were performed with primers in mouse exon 11 (145 bp; 5'-AGAGCGGAGCAAT-GGGTGGCAA-3' and 5'-GGCTGCGCAGTTGGCAGATGA-3') and rat exon 2 (185 bp; 5'-AATGTCCGCATAGACCCGAGCA-3' and 5'-CTGTAGACATAGGGTCCACGCT-3').

In situ hybridizations. *In situ* hybridizations were performed using ³⁵S sense and antisense RNA probes as described previously (Dolle and Duboule, 1989).

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