HNF1α and SREBP2 are important regulators of NPC1L1 in human liver

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Abstract Niemann-Pick C1-like 1 (NPC1L1), a key regulator of intestinal cholesterol absorption, is highly expressed in human liver. Here, we aimed to gain more insight into mechanisms participating in its hepatic regulation in humans. Correlation analysis in livers from Chinese patients with and without gallstone disease revealed strong positive correlations between NPC1L1 and sterol regulatory element binding protein 2 (SREBP2) (r = 0.74, P < 0.05) and between NPC1L1 and hepatic nuclear factor α (HNF4α) (r = 0.53, P < 0.05) mRNA expression. HNF4α is an upstream regulator of HNF1α; thus, we also tested whether HNF1α participates in the regulation of NPC1L1. We showed a dose-dependent regulation by SREBP2 on the NPC1L1 promoter activity and mRNA expression in HuH7 cells. Chromatin immunoprecipitation assay confirmed the binding of SREBP2 to the promoter in vivo. Surprisingly, HNF4α slightly decreased the NPC1L1 promoter activity but had no effect on its gene expression. By contrast, HNF1α increased the promoter activity and the gene expression, and an important HNF1 binding site was identified within the human NPC1L1 promoter. ChIP assays confirmed that HNF1α can bind to the NPC1L1 promoter in vivo.

Niemann-Pick C1-like 1 (NPC1L1) is a key regulator of intestinal cholesterol absorption and is supposed to be the target of the cholesterol-lowering drug ezetimibe (1, 2). Mice deficient in NPC1L1 have ~70% reduction in cholesterol absorption (1) and resistance to diet-induced hypercholesterolemia (3). NPC1L1 is widely expressed in many human tissues, with the highest expression in small intestine and in the liver (3, 4). In mice and rats, npc1l1 is predominantly expressed in the small intestine, whereas all others tissues showed expression levels <10% of the intestinal expression (1, 5). The exact function of NPC1L1 in the human liver is currently unknown. It was recently reported that NPC1L1 facilitates the uptake of free cholesterol from the culture medium in human (6) and rat (7) hepatoma cells. Previous reports also showed that NPC1L1 localizes to the canalicular membrane in hepatocytes (6, 8). Transgenic mice overexpressing human NPC1L1 in the liver had dramatically decreased biliary cholesterol concentration, which was returned to normal with ezetimibe treatment (8). This suggests that hepatic NPC1L1 could be another target of ezetimibe in humans.

Several genes involved in cholesterol synthesis and uptake are regulated by sterol regulatory element binding protein 2 (SREBP2). Activation of SREBP2 is dependent on the cholesterol status of the cell (9). When cellular cholesterol levels are low, SREBP2 is proteolytically cleaved to release the N-terminal portion to generate the mature form that can enter the nucleus and bind to sterol regulatory elements (SREs) or E-boxes in the promoter of various genes and affect gene expression (9, 10).

Hepatic nuclear factors (HNFs) 1 and 4 are expressed in various organs, including the liver, intestine, and pan-

Supplementary key words Niemann-Pick C1-like 1 • sterol regulatory element binding protein 2 • hepatic nuclear factor • cotransfection • chromatin immunoprecipitation • correlation analysis

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Abbreviations: ChIP, chromatin immunoprecipitation; GS, gallstone disease; GSF, gallstone-free; HNF, hepatic nuclear factor; NPC1, Niemann-Pick C1; NPC1L1, Niemann-Pick C1-like 1; SRE, sterol regulatory element; SREBP, sterol regulatory element binding protein.

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creases (11). Deficiency of HNF1α in mice (12) results in defect bile acid transport, increased bile acid and liver cholesterol synthesis, and impaired HDL metabolism. HNF4α knockout mice die before birth (13), and conditional liver-specific disruption of HNF4α (14) results in hepatomegaly, lipid deposition in the liver, reduced serum cholesterol and triglyceride levels, and elevated serum bile acid concentrations. Thus, both HNF1α and HNF4α play important roles in lipid homeostasis.

Since the physiological significance in human liver remains to be clarified, the aim of this study was to gain more insight into the mechanisms that participate in the transcriptional regulation of hepatic NPC1L1.

EXPERIMENTAL PROCEDURES

Materials

2×SYBRGreen Mastermix was purchased from MedProbe (Oslo, Norway). HuH7 and HEK293 cells were purchased from American Type Culture Collection (Manassas, VA). SREBP2, HNF1α, and IgG antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The human NPC1L1 promoter construct (or mutated constructs) and 2 µg pSV-β-galactosidase control vector (Promega, Madison, WI) with or without increasing concentrations of SREBP2, HNF1α, and HNF4α expression vectors or with 0.5 µg of each expression vector using Lipofectin reagent (Invitrogen, Carlsbad, CA) at a ratio of 3:1 (Lipofectin:DNA). pGL3 empty vector (Promega) was used to adjust for differences in amount of DNA added to the cells. Transfections of HEK293 cells were performed like those for HuH7 cells, except that Lipofectamine 2000 reagent (Invitrogen) at a ratio of 0.25:1 (Lipofectamine:DNA) was used. The pSV-β-galactosidase control vector was used to correct for variation in transfection efficiency. Forty-eight hours after transfection, cell lysates were prepared in reporter lysis buffer (Promega). β-Galactosidase and luciferase activities were determined using β-galactosidase and luciferase assay kits, respectively, according to the manufacturer’s instructions (Promega). All transfection data are expressed as luciferase activity corrected by β-galactosidase activity.

For gene expression analysis, HuH7 cells were transfected with 0, 0.05, 0.1, 0.5, 1, and 2 µg SREBP2, HNF1α, or HNF4α expression vector using Lipofectin reagent (Invitrogen). Forty-eight hours after transfection, total RNAs were prepared using TRIzol reagent according to the manufacturer’s protocol.

To study the effect of cholesterol on NPC1L1 gene expression, HuH7 cells were incubated for 12 h with 0, 0.1, 0.5, 1, and 2 mM LDL cholesterol or with 10% LPDS prior to RNA extraction. All cell experiments were performed in quadruplicates, and data represent means ± SEM.

Chromatin immunoprecipitation assay. The chromatin immunoprecipitation (ChIP) assays were performed using ~200 mg liver from a healthy donor as described (19). Specific antibodies for HNF1α (sc-6547×; Santa Cruz Biotechnology), SREBP2 (sc-8151×; Santa Cruz Biotechnology), and an IgG antibody (sc-2027; Santa Cruz Biotechnology), as a (baseline) control, were used (4 µg). Primers used for detection of the in vivo binding of SREBP2 to the two SREBP2 binding sites (SRE1 and SRE2) in the human NPC1L1 promoter were as follows: forward sequence (SRE1) 5′-GAAAGGGGAGGAGGCTGCTTTT-3′, and the reverse sequence 5′-TCAGGAAACCAAGGGCTGCTG-3′; and forward sequence (SRE2) 5′-CTAGGGGGTCGACCGGTGGGAC-3′, and the reverse sequence 5′-CTTCCTCCCTCTTGCCCTTGCTGC-3′. Primers used for detection of the in vivo binding of HNF1α to the human NPC1L1 promoter were as follows: forward sequence 5′-GAGAAGGAGGAGGCTGCTTTT-3′, and the reverse sequence 5′-TCAGGAAACCAAGGGCTGCTG-3′; and forward sequence (SRE2) 5′-CTAGGGGGTCGACCGGTGGGAC-3′, and the reverse sequence 5′-CTTCCTCCCTCTTGCCCTTGCTGC-3′. Primers used for detection of the in vivo binding of HNF1α to the human NPC1L1 promoter were as follows: forward sequence 5′-GAGAAGGAGGAGGCTGCTTTT-3′, and the reverse sequence 5′-TCAGGAAACCAAGGGCTGCTG-3′; and forward sequence (SRE2) 5′-CTAGGGGGTCGACCGGTGGGAC-3′, and the reverse sequence 5′-CTTCCTCCCTCTTGCCCTTGCTGC-3′. Also, in order to correct for different DNA loading (input versus immunoprecipitated samples), primers were designed using the human exon 7 of NPC1L1, with the forward sequence 5′-CCCAAGGAGGTTCCACATTTGG-3′ and the reverse sequence 5′-GAGAAGGAGGAGGCTGCTTTT-3′, and used as internal control in the PCR.

Electrophoretic mobility shift assay and supershift assay. Nuclear extracts were prepared from HuH7 cells as described by Azzout-Marniche et al. (20). Forward sequences for primers

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used to generate double-stranded probes were as follows: (−769 bp) 5′-CTCAAGGCTCCCAAGCCATGCTCCTGTC-3′; (−665 bp) 5′-AGCAGGACAAAGGAGATGTCCTGCA-3′; (−598 bp) 5′-CAGATTCTTCCCCTTTGCCTGACGAG-3′; (−360 bp) 5′-AGGCAGGATCTCCGCCAGCTGCTGTC-3′; (−158 bp) 5′-CAGTGGGAGTTGGCTACCCAGGAGCT-3′; (−119 bp) 5′-TCAGTTGTCCTAAACCCAGTCAGGGG-3′. The underlined bases in the forward sequences were deleted for the mutated HNF1 binding sites. Unlabeled double-stranded probes were generated by mixing 1 μg forward and 1 μg reverse oligonucleotide, 5 μl 1 M NaCl, and ddH2O up to 50 μl, and annealed at 95°C for 10 min. Labeled probes were generated by mixing 5 μl unlabeled probe, 2 μl 10× PNK buffer (Promega), 1 μl 0.1 M DTT, 1.5 μl T4 polynucleotide kinase (Promega), 2 μl [γ-32P]ATP, and ddH2O up to 50 μl and incubated at 37°C for 1 h, before passing through a Sephadex G-50 column (Amersham, Upsala, Sweden). The labeled probes (40,000 cpm) were combined with 10 μg nuclear extract along with 2 μg poly (dI-dC) (Amersham, Piscataway, NJ), 7.5 μl DNA binding buffer (80 mM HEPES-NaOH, pH 7.6, 0.2 M NaCl, 40 mM DTT, 2% v/v glycerol, 2 mM EDTA, and 12 μg/ml BSA), 1 μl 0.1 M DTT, 4 μl Buffer C (10 mM HEPES-KOH, pH 7.9, 0.4 M NaCl, 0.1 mM EDTA, 5% v/v glycerol, 1 mM DTT, and protease inhibitors), and ddH2O up to 30 μl. Binding reaction mixtures were incubated for 20 min at room temperature and resolved on a nondenatured (4% w/v) acrylamide gel in 1× TBE at 4°C for 3 h. For supershift assays, 2 μg HNF1α (sc-6547×; Santa Cruz Biotechnology) or 2 μg SREBP2 (sc-8151×; Santa Cruz Biotechnology) antibodies were added to the binding reaction mixtures. After electrophoresis, gels were dried and exposed over night to X-ray film at 80°C.

**Statistical analysis.** Data are expressed as means ± SEM for all cell experiments. Correlation analyses in patients were calculated by least square regression analysis. A P value of <0.05 was regarded as statistically significant. For cell experiments, multi-way ANOVA, followed by posthoc comparisons according to Dunnett test, was used with the exception of treatment of the cells with LPDS in which the Student’s t-test was used (Statistica software; Stat Soft, Tulsa, OK).

**RESULTS**

NPC1L1 correlates with SREBP2 and HNF4α in human livers

Gene expression analyses were performed in liver samples from 22 Chinese patients with cholesterol GS and 12 Chinese GSF patients. As we recently reported (18), no significant differences were observed in NPC1L1, SREBP2, and HNF4α mRNA expression between GS and GSF patients, though GS patients had 43% (P < 0.05) higher HNF4α mRNA expression compared with GSF patients.

A strong positive correlation between NPC1L1 and SREBP2 mRNA expression was present. As shown in Fig. 1A, when all patients were considered the regression coef-

**Fig. 1.** Correlation of mRNA expression in human liver from Chinese patients with and without cholesterol GS (n = 34): NPC1L1 and SREBP2 (A); HNF4α (B); HNF1α (C); PCSK9 (D), and between PCSK9 and SREBP2 (E) and HMG-CoA reductase and SREBP2 (F). Gene expressions were related to cyclophilin A mRNA. The correlation values were determined by least square regression analysis.
Regulation of NPC1L1 in human liver

The above observations prompted us to investigate whether SREBP2, HNF4α, and HNF1α may participate in the hepatic regulation of NPC1L1 in humans. Cotransfection experiments in human hepatoma cells (HuH7) using the human NPC1L1 promoter (−1,570 to +137 bp) and the SREBP2 expression vector were performed. A strong dose-dependent regulation by SREBP2 on the human NPC1L1 promoter activity (Fig. 2A) was observed. Also, the NPC1L1 mRNA expression increased ~60% (P < 0.05) using 0.5 µg SREBP2 expression vector (Fig. 2B). Activation of SREBP2 is dependent on the free cholesterol levels inside the cell. To study the effect on the endogenous NPC1L1 gene expression under more physiological conditions, HuH7 cells were depleted or loaded with cholesterol. Loading of the cells with LDL cholesterol decreased both the NPC1L1 and the SREBP2 mRNA expression (Fig. 2C). A strong positive correlation (r = 0.94, P < 0.05) between NPC1L1 and SREBP2 mRNA expression was also observed (data not shown). Cholesterol depletion, on the other hand, resulted in an insignificant trend toward increased NPC1L1 mRNA levels (data not shown). Recently, Alrefai et al. (23) identified two SREs, SRE1 and SRE2, in the NPC1L1 promoter. Two SREBs, SRE1 and SRE2, in the NPC1L1 promoter. The IgG antibody was used as a baseline control and used to compare the relative fold enrichment of the NPC1L1 promoter by the specific DNA fragments. Before immunoprecipitation, a small aliquot of chromatin was saved and used as an input control. Experiments were performed in triplicate, and data are expressed as means ± SEM.

ANOVA followed by posthoc comparisons according to the Dunnett test. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. D: Soluble chromatin (−60 µg DNA, −500 bp in length) were prepared from human liver and immunoprecipitated with 4 µg antibodies against SREBP2 or IgG and amplified by real-time RT-PCR using primers for detection of SRE1 (−91/−81 bp) and SRE2 (−748/735 bp) in the NPC1L1 promoter. The IgG antibody was used as a baseline control and used to compare the relative fold enrichment of the NPC1L1 promoter by the specific DNA fragments. Before immunoprecipitation, a small aliquot of chromatin was saved and used as an input control. Experiments were performed in triplicate, and data are expressed as means ± SEM.
the human NPC1L1 promoter. To determine whether SREBP2 can bind to these SREs in the NPC1L1 promoter in vivo, we performed ChIP assay using human liver. Immunoprecipitation with a specific antibody against SREBP2 and primers designed to target the two previously identified SREs were used in the PCR analysis. This led to >36-fold enrichment of SRE1 (−91/−81 bp) and ~17-fold enrichment of SRE2 (−748/−738 bp) sequences present in the human NPC1L1 promoter (Fig. 2D). Collectively, these results show that SREBP2 can regulate and bind to the NPC1L1 promoter in human liver.

Effects of HNF4α overexpression on NPC1L1 promoter activity and gene expression

Due to the positive correlation between NPC1L1 and HNF4α in the patients, we also performed cotransfections in HuH7 cells using the human NPC1L1 promoter along with the HNF4α expression vector. Surprisingly, HNF4α decreased the human NPC1L1 promoter activity in a dose-dependent fashion (Fig. 3A), whereas HNF4α overexpression had no effect on its mRNA expression (Fig. 3B). Iwayanagi, Takada, and Suzuki (24) showed that the transcription of NPC1L1 was stimulated by HNF4α together with SREBP2, but not by HNF4α alone. Cotransfection of HuH7 cells with both SREBP2 and HNF4α expression vectors was performed to test a possible synergism in the activation of the NPC1L1 promoter. However, no further activation of the promoter activity occurred (Fig. 3C).

HNF1α interacts with the human NPC1L1 promoter in vitro and in vivo

HNF4α is an essential positive regulator of HNF1α (25). HNF4α and HNF1α can bind directly to one another, and both contain a binding site in their promoter region for the other (26), suggesting a reciprocal regulation. Thus, we wanted to test whether HNF1α might participate in the regulation of NPC1L1. We performed cotransfections in HuH7 cells using the human NPC1L1 promoter along with the HNF1α expression vector. These experiments revealed a dose-dependent regulation by HNF1α on the NPC1L1 promoter activity (Fig. 4A) and increased mRNA expression (Fig. 4B). Next, we screened the sequence using TESS to search for putative HNF1 binding sites in the human NPC1L1 promoter. Six HNF1 cis-elements were found located −119/−114, −158/−144, −360/−354, −598/−593, −665/−660, and −769/−763 bp upstream of the ATG start codon (Fig. 5). We performed mutagenesis on these HNF1 cis-elements and used the mutant constructs in transfection experiments with or without the HNF1α expression vector. These experiments showed that mutation of one HNF1 binding site (−158/−144) almost completely abolished the regulatory effect of HNF1α on the NPC1L1 promoter activity (Fig. 4C). However, muta-
ANOVA followed by posthoc comparisons according to the Dunnett test. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. D: Soluble chromatin (~60 µg DNA, ~500 bp in length) were prepared from human liver and immunoprecipitated with 4 µg antibodies against HNF1α or IgG and amplified by real-time RT-PCR using primers to target the region spanning over the six HNF1 binding sites (~769 to −119 bp) in the NPC1L1 promoter. The IgG antibody was used as a baseline control and used to compare the relative fold enrichment of the NPC1L1 promoter by the specific DNA fragments. Before immunoprecipitations, a small aliquot of chromatin was saved and used as an input control. Experiments were performed in triplicate, and data are expressed as means ± SEM.

**DISCUSSION**

In this study, we investigated whether SREBP2, HNF4α, and HNF1α might be important regulators of the NPC1L1 promoter in human liver in vitro and in vivo.

SREBP2 regulates genes involved in cholesterol biosynthesis (e.g., HMG-CoA reductase and uptake (e.g., LDL-receptor), and it is activated upon low cellular cholesterol levels (9). Sané et al. (4) reported that SREBP2 mRNA expression was increased in Caco-2 cells deprived of NPC1L1. Alrefai et al. (23) reported that NPC1L1 mRNA expression was increased in Caco-2 cells deprived of NPC1L1. Regulation of NPC1L1 in human liver.
which had no effect on the promoter activity in our experiments. In the cotransfection experiments, we used 0.5 \( \mu g \) of each expression vector, whereas Iwayanagi, Takada, and Suzuki used 0.1 \( \mu g \) SREBP2 and 0.5 \( \mu g \) HNF4 expression vector decreased by cholesterol loading and increased in response to cholesterol depletion in Caco-2 cells, and two SREs in the NPC1L1 promoter region were identified. These studies suggest that low levels of cholesterol activate the transcription of SREBP2, leading to an upregulation of the LDL-receptor and HMG-CoA reductase, which in turn increases NPC1L1 expression with a subsequent increase in cholesterol absorption. Furthermore, in miniature pigs treated with a combination of ezetimibe plus simvastatin, Telford et al. (27) reported increased NPC1L1 expression in liver and intestine, which was positively correlated with the SREBP2, HMG-CoA reductase, and LDL receptor expressions. In line with previous reports, we showed a) strong positive correlation between NPC1L1 and SREBP2 mRNA expression in liver from Chinese patients with and without gallstone, as well as divided by the presence of gallstone disease; b) a strong dose-dependent regulation of SREBP2 on the NPC1L1 promoter activity in HuH7 cells and that this finding is liver specific (>13-fold in HuH7 vs. <50% in HEK293 cells using 0.5 \( \mu g \) SREBP2 expression vector; Fig. 3D); c) increased NPC1L1 mRNA expression following transfection with SREBP2 expression vector; d) decreased NPC1L1 and SREBP2 mRNA expression following cholesterol loading of the cells with LDL; and, finally, e) binding of SREBP2 to the human NPC1L1 promoter in human liver. The in vivo binding of SREBP2 to NPC1L1 may thus participate in a coordinated regulation wherein hepatocytes may acquire more cholesterol. Thus, SREBP2 seems to be an important regulator of both the intestinal and the hepatic NPC1L1 expression in humans.

Misawa et al. (28) reported a direct interaction between SREBP2 and HNF4\( \alpha \) to enhance the sterol isomerase gene expression in Caco-2 and HepG2 cells. Iwayanagi, Takada, and Suzuki (24) showed that HNF4\( \alpha \) small interfering RNA reduced the mRNA expression and abolished the cholesterol-dependent regulation of NPC1L1 in HepG2 cells; also, the transcription of NPC1L1 was stimulated by HNF4\( \alpha \) together with SREBP2, but not by HNF4\( \alpha \) alone. In our study, cotransfection with the human NPC1L1 promoter along with HNF4\( \alpha \) expression vector slightly decreased the promoter activity but had no effect on the NPC1L1 gene expression. Also, no synergistic activation of NPC1L1 promoter activity was seen in HuH7 cells after cotransfection with SREBP2 and HNF4\( \alpha \). The discrepancies might be due to different amounts of expression vector; we used up to 2 \( \mu g \), whereas Iwayanagi, Takada, and Suzuki (24) used 0.5 \( \mu g \) of HNF4\( \alpha \) expression vector.
vector. Also, different plasmids were used to create the HNF4α expression vectors. Furthermore, we used the human hepatoma cell line HuH7, whereas Iwayanagi, Takada, and Suzuki (24) used HepG2 cells.

The strong correlation between NPC1L1 and HNF4α mRNA expression in our Chinese patients suggests that HNF4α may function by transactivating NPC1L1 via binding to other transcription factors, including SREBP2. HNF4α is an upstream regulator of HNF1α, and both contain binding sites for each other in their promoter region (26, 29), suggesting a reciprocal regulation. HNF1α was recently shown to be an important regulator of ACAT2 (19), which is another important gene involved in cholesterol homeostasis. By coimmunoprecipitation of nuclear extracts from human liver with an antibody raised against HNF1α and detection by Western blot with an HNF4α antibody, we were able to show that a protein-protein interaction between HNF4α and HNF1α occurs in human liver (50). Thus, it is possible that HNF4α also exerts its action on NPC1L1 by trans-activating NPC1L1 via binding to HNF1α or by increasing its expression. Interestingly, Odom et al. (29) reported that HNF4α can bind to the NPC1 gene but not to NPC1L1 in primary human hepatocytes. The NPC1L1 protein shares 42% identity and 51% similarity with NPC1 (31); despite this, mice deficient in NPC1 did not show altered functional activity of NPC1L1 implying their independent roles and regulations (32).

Odom et al. (29) also reported that HNF4α can bind to the NPC1L1 gene in primary human hepatocytes. This is in line with our results in HuH7 cells, showing a dose-dependent regulation of HNF1α on the NPC1L1 promoter activity and increased mRNA expression following HNF1α overexpression. The liver specificity of this finding was tested by cotransfection of HEK293 cells with the human NPC1L1 promoter and 0.5 μg HNF1α expression vector (Fig. 3D); no induction by HNF1α on the promoter activity was identified in these cells. Also, we identified an HNF1 binding site located −158/−144 bp upstream of transcription start site and showed that mutation of this site almost completely abolished the induction by HNF1α. Electrophoretic mobility shift assay and supershift assays showed a direct binding of HNF1α to this site but not to the other five cis-elements. To show the in vivo binding of HNF1α to the NPC1L1 promoter in human liver, we performed ChIP analysis. The six putative HNF1 binding sites (located at −119, −158, −360, −598, −665, and −769 bp upstream of the ATG start codon) are closely located in the NPC1L1 promoter region, and the sonication procedure in the ChIP analysis generally generates DNA fragments of ~500–800 bp. Thus, primers designed to span over the six HNF1 binding sites were used in the PCR analysis and revealed that HNF1α can bind to the human NPC1L1 promoter in vivo. Collectively, these data suggest that HNF1α may be an important regulator of the hepatic expression of NPC1L1 in humans.

In summary, in this study, we showed that SREBP2 and HNF1α are important transcription factors for the hepatic NPC1L1 promoter activity that can bind to and regulate its expression in humans.

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