Journal of Steroid Biochemistry & Molecular Biology 132 (2012) 41–47

Contents lists available at SciVerse ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

# Involvement of multiple elements in FXR-mediated transcriptional activation of *FGF19*

Masaaki Miyata[a,*], Tatsuya Hata[a], Hiroki Yamakawa[a], Tatehiro Kagawa[b], Kouichi Yoshinari[a], Yasushi Yamazoe[a]

[a] *Division of Drug Metabolism and Molecular Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan*
[b] *Department of Gastroenterology, Tokai University, School of Medicine, Isehara, Japan*

ARTICLE INFO

*Article history:*
Received 17 January 2012
Received in revised form 20 April 2012
Accepted 23 April 2012

*Keywords:*
FGF19
FXR
PXR
Bile acid
GW4064

ABSTRACT

The intestinal endocrine hormone human fibroblast growth factor 19 (FGF19) is involved in the regulation of not only hepatic bile acid metabolism but also carbohydrate and lipid metabolism. In the present study, bile acid/farnesoid X receptor (FXR) responsiveness in the *FGF19* promoter region was investigated by a reporter assay using the human colon carcinoma cell line LS174T. The assay revealed the presence of bile acid/FXR-responsive elements in the 5′-flanking region up to 8.8 kb of *FGF19*. Deletion analysis indicated that regions from −1866 to −1833, from −1427 to −1353, and from −75 to +262 were involved in FXR responsiveness. Four, four, and two consecutive half-sites of nuclear receptors were observed in the three regions, respectively. An electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) assay revealed FXR/retinoid X receptor α (RXRα) heterodimer binding in these three regions. EMSA and reporter assays using mutated constructs indicated that the nuclear receptor IR1, ER2, and DR8 motifs in the 5′-flanking region were involved in FXR responsiveness of *FGF19*. Lithocholic acid (LCA) (10 μM), chenodeoxycholic acid (CDCA) (10 μM), or GW4064 (0.1 μM) treatment increased reporter activity in a construct including the three motifs under FXR-expressing conditions whereas LCA and not CDCA or GW4064 treatment increased the reporter activity under pregnane X receptor (PXR)-expressing conditions. These results suggest that *FGF19* is transcriptionally activated through multiple FXR-responsive elements in the promoter region.



## 1. Introduction

Farnesoid X receptor (FXR) is a bile acid-activated nuclear receptor that regulates bile acid homeostasis. Many studies suggest that FXR is involved in the regulation of lipid and carbohydrate metabolism [1–4]. It directly regulates the expression of genes involved in hepatic bile acid, lipid, and carbohydrate metabolism. In contrast, recent studies have highlighted the role of intestinal FXR-dependent fibroblast growth factor 19 (FGF19) signaling in the regulation of hepatic bile acid, lipid, and carbohydrate metabolism [5,6].

FGF19 is mainly expressed in the intestine and acts on the liver as an endocrine hormone, regulating various metabolic processes that involve bile acids, lipids, and carbohydrates [7–13]. Previous reports have suggested that FGF19 plays an important role in the regulation of bile acid homeostasis by downregulating the hepatic expression of CYP7A1, a rate-limiting enzyme in hepatic bile acid synthesis [7,8]. In addition, it suppresses the excretion of bile acids into the small intestine through gallbladder filling [14]. Studies on FGF19 transgenic mice and FGF19-treated mice have indicated that FGF19 lowers the hepatic and serum levels of lipids such as triglycerides and free fatty acids [15–17]. Recent studies have also reported that FGF19 activates a physiologically important, insulin-independent endocrine pathway that enhances hepatic protein and glycogen synthesis and suppresses gluconeogenesis in the liver [11,13]. Unlike insulin, FGF19 does not stimulate lipogenesis.

Patients with metabolic syndrome have 65% lower FGF19 serum levels than healthy individuals, suggesting that serum FGF19 is an independent marker of metabolic syndrome [18]. Serum FGF19 levels increase on intake of chenodeoxycholic acid (CDCA) [19];

*Abbreviations:* CA, cholic acid; ChIP, chromatin immunoprecipitation; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; EMSA, electrophoretic mobility shift assay; FGF19, fibroblast growth factor 19; FXR, farnesoid X receptor; LCA, lithocholic acid; PCR, polymerase chain reaction; PXR, pregnane X receptor; RXRα, retinoid X receptor α.

* Corresponding author at: Division of Drug Metabolism and Molecular Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University, 6-3, Aoba, Aramaki, Aoba-ku, Sendai 980-8578, Japan. Tel.: +81 22 795 6829; fax: +81 22 795 6826.
*E-mail address:* miyata@m.tohoku.ac.jp (M. Miyata).

0960-0760/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.jsbmb.2012.04.008

however, these levels decrease in patients with primary bile acid diarrhea, which results in increased hepatic bile acid synthesis [20]. *FGF19* mRNA is not usually present in the human liver [21,22]. However, hepatic *FGF19* mRNA levels are markedly elevated in patients with cholestasis [23]. *FGF19* mRNA levels in primary human hepatocytes are induced by treatment with CDCA or GW4064, an FXR-specific ligand. However, little is known about bile acid/FXR-mediated intestinal FGF19 expression.

In the present study, we investigated bile acid/FXR responsiveness in the *FGF19* promoter region by an *in vitro* reporter assay using the human colon adenocarcinoma cell line LS174T. We identified novel bile acid/FXR-responsive elements in the *FGF19* promoter region.

## 2. Materials and methods

### 2.1. Materials

T4 polynucleotide kinase and restriction enzymes were purchased from New England BioLabs (Ipswich, MA). Fetal calf serum and [$\gamma$-$^{32}$P]ATP were purchased from Nichirei (Tokyo, Japan) and Perkin-Elmer Life and Analytical Sciences (Waltham, MA), respectively. CDCA, lithocholic acid (LCA), deoxycholic acid (DCA), and cholic acid (CA) were purchased from Sigma-Aldrich (St. Louis, MO). GW4064 was provided by Dr. Timothy M. Wilson (GlaxoSmithKline, Research Triangle Park). All other chemicals were purchased from Wako Pure Chemicals (Osaka, Japan).

### 2.2. Plasmid preparation

FGF19 luciferase constructs were prepared using DNA fragments of the *FGF19* promoter (−8810 to +262) and the second exon, second intron, and third exon (+848 to +5200), which were amplified by polymerase chain reaction (PCR) using KOD FX (Toyobo, Osaka, Japan) with human genomic DNA as a template. The fragments were inserted upstream or downstream of the luciferase gene of pGL4.10 (Promega, Madison, WI). A mutation construct was prepared using the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

### 2.3. Cell culture

LS174T cells were obtained from the Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). These cells were cultured in minimum essential medium containing 10% fetal bovine serum, nonessential amino acids (Invitrogen, Carlsbad, CA), and penicillin–streptomycin (Invitrogen).

### 2.4. Analyses of mRNA levels

Total RNA was prepared from LS174T cells using the RNAgents Total Isolation System (Promega, Madison, WI). RNA concentration was determined by measuring the absorbance at 260 nm using a DU800 spectrophotometer (Beckman Coulter, Fullerton, CA). Single-stranded cDNA was synthesized using an oligo(dT) primer and the Ready-To-Go You-Prime First-Strand Beads Kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). These cDNA templates were subjected to real-time quantitative PCR (qPCR) using SYBR Green 1 with the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Relative mRNA levels were calculated by the comparative threshold cycle method. The following specific forward and reverse primers were used for real-time qPCR: *FGF19*, sense, 5′-CACGGGCTCTCCAGCTGCTTCCTGCG-3′ and antisense, 5′-TCCTCCTCGAAAGCACAGTCTTCCTCCG-3′; *GAPDH*, sense, 5′-AACAGCCTCAAGATCATCAGC-3′ and antisense, 5′-GGATGATGTTCTGGAGAGCC-3′.

### 2.5. Reporter assay

The LS174T cells were maintained as described previously [24]. The cells were seeded in a 48-well plate at $3 \times 10^4$ cells/well for 24 h before transfection. The reporter construct pTarget or pTarget/hFXR and pRL-SV40 were cotransfected by the calcium phosphate method using the Cellphect Transfection Kit (GE Healthcare). The cells were washed with phosphate-buffered saline 12 h after transfection and then treated with compounds (GW4064, 0.1 μM; bile acids, 10 μM) or vehicle (0.1% DMSO) in fetal bovine serum-free medium for 24 h. Thereafter, the cells were harvested and dual-luciferase activities were determined using the Dual-Luciferase Reporter Assay System (Promega) as described previously [25].

### 2.6. Electrophoretic mobility shift assay (EMSA)

Human FXR and retinoid X receptor α (RXRα) proteins were synthesized *in vitro* from pTNT/hFXR and pTNT/hRXRα, respectively, using the TNT Quick Coupled Transcription/Translation System (Promega). EMSA was performed as described previously [26]. Double-stranded oligonucleotide probes were labeled with [$\gamma$-$^{32}$P]ATP using T4 polynucleotide kinase and purified with NICK columns (GE Healthcare). The binding reaction was performed in a reaction mixture (15 μl) containing 10 mM Tris–HCl (pH 8.0), 5% glycerol, 100 mM KCl, 1 mM dithiothreitol, 1 μg of poly(dI–dC) (GE Healthcare), and 1 μl each of synthesized FXR- and/or RXRα-containing lysate or control lysate. Reaction mixtures were preincubated on ice for 30 min before the addition of the $^{32}$P-labeled probe. The samples were kept at room temperature for an additional 30 min and then separated using a 4% polyacrylamide gel in 0.25 × Tris–boric acid–EDTA buffer at 40 mA. The gel was dried and exposed to an imaging plate to detect DNA–protein complexes using Imaging Analyzer FLA-3000 (Fujifilm, Tokyo, Japan). For certain reactions, 200 ng of antihuman FXR mouse monoclonal antibody (A9033; Perseus Proteomics, Tokyo, Japan), excessive unlabeled probe, or oligonucleotide IR1 probe (CCTGCAGTTCAGTGACCTTTGCA) was added to the reaction mixture before incubation.

### 2.7. Chromatin immunoprecipitation assay

The LS174T cells were seeded and simultaneously transfected with pTarget/hFXR using jetPEI (Funakoshi, Tokyo, Japan). Sixty-seven hours after transfection, the cells were treated with 0.1 μM GW4064 for 5 h. Chromatin immunoprecipitation (ChIP) assays were performed as described previously [27,28] with minor modifications. After crosslinking with 1% formaldehyde and sonication of the cells, supernatant was obtained by centrifugation, and a portion of the supernatant was retained as the input sample. The supernatant was incubated with protein G-coupled Dynabeads (Invitrogen) at 4 °C for 1 h, and the beads were then removed. The supernatant was then incubated with normal mouse IgG (Millipore, Billerica, MA) or antihuman FXR mouse monoclonal antibody (A9033; Perseus Proteomics, Tokyo, Japan) at 4 °C overnight. Protein G-coupled Dynabeads was added to the reaction and the mixture was further incubated at 4 °C for 4–6 h to immunoprecipitate the chromatin–antibody complex. The bound chromatin was eluted and incubated at 65 °C overnight to decrosslink. After treatment with RNase A (Nacalai Tesque, Kyoto, Japan) and proteinase K (Sigma-Aldrich), genomic DNA was purified with Wizard SV gel and PCR using KOD FX Neo (Toyobo). The primers used for the P1-containing region (from −1938 to −1758) were 5′-AGTGCTACATCCCAGGCCCTCTCTG-3′ and 5′-TGTGCTGCGTCTTATCCCGGGACAG-3′. The primers for the P2-containing region (from −1519 to −1330) were