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Transcription of the human microsomal epoxide hydrolase gene (EPHX1) is regulated by an HNF-4α/CAR/RXR/PSF complex☆

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ABSTRACT

Microsomal epoxide hydrolase (mEH) is a bifunctional protein that plays a central role in the metabolism of numerous xenobiotics as well as mediating the sodium-dependent transport of bile acids into hepatocytes where they are involved in cholesterol excretion and metabolism, lipid digestion and regulating numerous signaling pathways. Previous studies have demonstrated the critical role of GATA-4 and a C/EBPα–NF/Y complex in the regulation of the mEH gene (EPHX1). In this study we show that HNF-4α and CAR/RXR also bind to the proximal promoter region and regulate EPHX1 expression. Bile acids, which inhibit the expression of HNF-4α also decrease the expression of EPHX1. Studies also established that the binding of HNF-4α was essential for the activation of EPHX1 activity by CAR suggesting the formation of a complex between these adjacent factors. The nature of this regulatory complex was further explored using a biotinylated oligonucleotide of this region in conjunction with BioMag beads and mass spectrometric analysis which demonstrated the presence of an additional inhibitory factor (PSF), confirmed by co-immunoprecipitation and ChIP analyses, which interacted with DNA-bound CAR/RXR/HNF-4α forming a 4-component regulatory complex.

1. Introduction

Microsomal epoxide hydrolase (mEH) is a bifunctional protein that is expressed on the hepatocyte endoplasmic reticulum membrane, where it is involved in the metabolism of numerous xenobiotics [1], and on the sinusoidal plasma membrane where it can mediate the sodium-dependent transport of bile acids into hepatocytes [2–10]. The bile acids play a critical role in the regulation of cholesterol metabolism, signal transduction pathways, digestion of dietary lipids and excretion of numerous metabolized xenobiotics [11,12]. Naturally occurring mutations in the human mEH gene (EPHX1) have been shown to result in a large decrease in mEH protein expression associated with a significant decrease in bile acid uptake resulting in highly elevated serum bile acid levels (hypercholanemia) [10]. The expression of mEH is also dramatically altered during development [7]. In previous studies we have demonstrated that GATA-4 [13] and a C/EBPα–NF/Y complex [14] play a critical role in regulating the constitutive expression of EPHX1.

Analysis of the EPHX1 proximal promoter region (E1) (−80/+25 bp) identified a putative binding site for hepatocyte nuclear factor 4α (HNF-4α, NR2A1) and a DR-4 motif. An alternate promoter (E1-b) has been reported that regulates EPHX1 expression in other human tissues [15] HNF-4α is expressed in the liver and binds to DR-1-like motifs as a homodimer. Studies have established this factor to be a critical transcription regulator for many genes specifically expressed in the liver [16–18]. The constitutive androstane receptor (CAR), a member of the nuclear hormone receptor superfamily, can bind to a DR-4 motif where it heterodimerizes with retinoid X receptor (RXR) and activates the expression of numerous genes involved in Phase I and Phase II biotransformation and the expression of membrane transport proteins [19,20]. Several studies also have demonstrated that there is cross talk between these factors where HNF-4α is critical for the expression of CAR activity [21,22]. Further analysis of this putative regulatory complex by affinity chromatography using a biotinylated oligonucleotide and mass spectrometry indicated the presence of polypyrimidine tract binding protein-associated splicing factor (PSF) which is a multifunctional protein that is involved in pre-mRNA splicing as well as transcriptional regulation [23] and has been shown to interact with several regulatory factors [24–26] and corepress several nuclear receptors such as the androgen [27] and progesterone receptors [28]. In this study we have demonstrated that EPHX1 transcription is regulated in part by the interaction of PSF with a DNA bound CAR, RXR, and HNF-4α complex.

Abbreviations: mEH, microsomal epoxide hydrolase; EPHX1, microsomal epoxide hydrolase gene; CAR, constitutive androstane receptor; RXR, retinoid X receptor; HNF-4α, hepatocyte nuclear factor-4α; SHP, small heterodimer partner; CDCA, chenodeoxycholic acid; PSF, polypyrimidine tract binding protein-associated splicing factor; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation

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2. Material and methods

2.1. Plasmids

The −80/+25 EPXH1 core promoter fragment was inserted into the promoterless expression vector pGL3 (Promega) containing the firefly luciferase reporter gene. The construct containing a mutated HNF-4α binding site was prepared by two polymerase chain reactions (PCR) using the −80/+25 EPHX1 construct as a template. The first PCR step used the 5′ primer, 5′-AGCAAGCTTTGTATCTCTCAGGTCAGC-3′ containing a HindIII site (underlined) and a mutant 3′ primer, 5′-GG A**GAA**T**TC**ACAGCAAACTGAACCACG-3′, containing an EcoRI site (underlined). The mutated nucleotides are in bold face. The second PCR used the following primers: 5′ primer, 5′-TGTG**AATTC**TCCAGG GGAGATAACCAC which contains an EcoRI site (underlined) and 3′ primer, 5′-ATCGGATCCAATTGCACAGTCCTGCCAAGTCAG, which contains a BamHI site (underlined). The first PCR product was digested with HindIII and EcoRI and the second PCR product was digested with EcoRI and BamHI. In order to insert the mutated DNA fragment into pGL3, we modified the linker sequence where the restriction endonuclease sites were changed from KpnI–SacI–MluI–NheI–SmaI–XhoI–BglII–HindIII to HindIII–SmaI–XhoI–BglII–EcoRI by ligation between the pGL3 vector digested with KpnI and HindIII and the new linker sequence formed by annealing two synthetic oligonucleotides: 5′-AAGCTTCCCGGGCTCGAGA TCTGAATTC-3′ and 5′-AGCTGAATTCAGATCTCGAGCCCGGGAAGCTTGT AC-3′, which formed a sticky end for the KpnI and HindIII at upstream and downstream end, respectively. The modified pGL3 vector was digested with HindIII and BglII, and with the two digested PCR products in a three-way ligation. To prepare a core promoter construct with mutated DR-4 motif, PCR was performed with a 5′ primer 5′-AGCAAGCTTTGTATCTCTCA**CTG**CAGCGTGGTTCAGTTTGCTGTG3′, which contains a HindIII site (underlined) and a 3′ primer 5′-ATCGGATCC AATTGCACAGTCCTGCCAAGTCAG, which contains a BamHI site (underlined). The mutated nucleotides are in bold face. The PCR product was digested with HindIII and BamHI and inserted into the modified pGL3 vector digested with HindIII and BglII. The construct in which both the HNF-4α and the DR-4 sites are mutated was prepared by PCR using the same primers for the single DR-4 mutant, using the HNF-4α mutant as the template.

The expression vector for human HNF-4α (pcDNA-3) was provided by Dr. Akiyoshi Fukamizu (University of Tsukuba, Tsukuba, Japan). The expression plasmid for the dominant negative form of HNF-4α was obtained from Dr. Ryuichiro Sato (University of Tokyo, Tokyo, Japan). The expression vectors for CAR and PXR (pCR3) were obtained from Dr. Thomas Kocarek (Wayne State University, Detroit, MI). The expression vector for SHP (pCMX) was provided by Dr. David Mangelsdorf (University of Texas Southwestern Medical Center, Dallas TX). The expression vector for PSF (pcDNA3.1) was obtained from Dr. Herbert H. Samuels (New York University School of Medicine, New York). All plasmids were sequenced prior to use.

2.2. Promoter activity assay

HepG2 and HeLa cells were obtained from the American Type Culture Collection (ATCC) and grown at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The wild type and mutant EPHX1 promoter constructs (−80/+25) were transiently transfected into HepG2 or HeLa cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Renilla luciferase reporter plasmid was cotransfected as an internal control to monitor transfection efficiency. The experiment was carried out in triplicate in 24-well plates and repeated at least three times. Forty-eight hours after transfection, the reporter luciferase activities were measured with the Dual-Glo Luciferase Assay System to measure both firefly and Renilla luciferase activities. Whenever expression vectors were used, empty vector was added in the control samples to keep total amount of added DNA constant (0.8 μg for each well). HepG2 cells containing the WT EPHX1 promoter construct were also transfected with plasmids for HNF-4α, CAR, PSF as well as control and siRNAs for HNF-4α (Invitrogen), CAR (Invitrogen) or PSF shRNA (Santa Cruz Biotechnology).

2.3. Electrophoresis mobility shift assay (EMSA)

Nuclear extracts were prepared from cultured HepG2 cells as previously described [13]. Sense and antisense oligonucleotides were synthesized, annealed and radiolabeled with ^{32}P-dCTP in fill-in reactions with the Klenow large fragment of DNA polymerase I. The sequence of the sense strand is given below. The WT probe corresponding to the EPHX1 −55/−31 bp fragment containing the binding site for HNF-4α (underlined) was 5′-AGTTTGCTGTGCAGAGTCCAGGGGA-3′. The mutant probe where the HNF-4α site was destroyed (M) was 5′-AGTTTGCT GTG**AATTC**TCCAGGGGA-3′ (mutated nucleotides in boldface). The WT probe corresponding to the EPHX1 −76/−50 bp fragment containing the CAR binding site (DR-4 underlined) was 5′-TGTCTCAGGTCAGC GTGGTTCAGTTTG-3′. The mutant probe where the CAR site is mutated (M) was 5′-TGTCTCA**CTG**CAGCGTGGTTCAGTTTG-3′. The probe containing both the CAR (DR-4) motif and the HNF-4α binding site was from −74/−31 bp. The sense strand was 5′-TCTC-AGGTCAGCG TGGTTCAGTTTGCTGTGCAGAGTCCAGGGGA-3′. In super-shift assays, antibodies against HNF-4α, CAR and RXR were purchased from Santa Cruz Biotechnology, Inc. and incubated with the reaction mixture on ice for 2 h before loading on the gel. The EMSA was carried out as described previously [13] and the protein-DNA bands were visualized by autoradiography.

2.4. Co-immunoprecipitation assay and Western blotting

HepG-2 nuclear extracts (300 μg) were incubated with 15 μl of protein A/G-agarose beads and 8 μg anti-PSF or HNF-4α antibodies as previously described. Pre-immune IgG was used as a negative control. Eluted proteins were separated by SDS-PAGE, electrophoretically transferred to a polyvinylidine difluoride membrane (Millipore). Proteins were detected with the indicated antibodies in a dilution of 1:100 to 1:500 with Western Breeze Chromogenic Western Blot Immunodetection Kit (Invitrogen). Subconfluent HepG2 cells with and without small heterodimer partner (SHP) treatment were harvested by trypsinization and lysed with universal lysis/immunoprecipitation buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 25 mM NaF, 25 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.1 mM PMSF, 5 μg of leupeptin/ml, 0.5%(v/v) Triton X-100, 0.5% (v/v) Nonidet P-40). Protein concentration was determined by the Bradford method (Bio-Rad). Western blotting was performed as described above.

2.5. Chromatin immunoprecipitation (ChIP)

HepG2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum to 80% confluency in a 15 cm plate and chromatin was prepared using ChIP-IT Express Kit (Active Motif) according to the manufacturer's recommendation. All the buffers and reagents described below were provided by the manufacturer. Briefly, cells were fixed in 20 ml minimal cell culture medium containing 1% formaldehyde for 10 min and fixation was stopped by Glycine-Stop Fix solution and cells were scrapped and collected by centrifugation and lysed in lysis buffer for 30 min on ice. Cells were then dounce homogenized and nuclei were collected by centrifugation. Nuclei were resuspended in digestion buffer and chromatin was optimally sheared to yield bands between 100 and 200 bp as assessed by agarose gel analysis, by adding Enzymatic Shearing Cocktail and incubated for 8–10 min at 37 °C. Sheared chromatin was collected by centrifugation at 15,000 rpm for 10 min at 4 °C. Immunoprecipitation was performed by using 7 μg of sheared chromatin, 3 μg of specific antibodies