Archives of Biochemistry and Biophysics 517 (2012) 123–130

Contents lists available at SciVerse ScienceDirect

Archives of Biochemistry and Biophysics

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

Transactivation of *ABCG2* through a novel *cis*-element in the distal promoter by constitutive androstane receptor but not pregnane X receptor in human hepatocytes

Satoshi Benoki, Kouichi Yoshinari *, Tsubasa Chikada, Jun Imai, Yasushi Yamazoe

Division of Drug Metabolism and Molecular Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University, 6-3 Aramaki-aoba, Aoba-ku, Sendai, Miyagi 980-8578, Japan

ARTICLE INFO

Article history:
Received 16 July 2011
and in revised form 6 October 2011
Available online 10 November 2011

Keywords:
ABC transporter
CAR
Nuclear receptor
PXR
Transcriptional regulation
Xenobiotic induction

ABSTRACT

A previous report demonstrated that treatment of human hepatocytes with phenobarbital, an activator of nuclear receptor constitutive androstane receptor (CAR), increases mRNA levels of an efflux transporter ABCG2, which is involved in the excretion of xenobiotics in liver and intestine. The results suggest that human CAR (hCAR) transactivates human *ABCG2* (*hABCG2*) expression. In this study, we confirmed increase in *ABCG2* mRNA levels in human hepatocytes after adenoviral expression of hCAR and treatment with its activator. Reporter assays suggested the existence of an hCAR-responsive element between −8000 and −7485 of *hABCG2* promoter. Electrophoretic mobility shift assays and chromatin immunoprecipitation assays identified a DR5 motif (direct repeat separated by five nucleotides) within the region as a binding motif of hCAR/human retinoid X receptor α heterodimer. The introduction of mutations into the DR5 motif resulted in the complete loss of the hCAR-mediated transactivation. Interestingly, human pregnane X receptor, belonging to the same NR1I subfamily as CAR, did not activate any reporter gene containing the DR5 motif. Taken together, our present findings suggest that hCAR transactivates *hABCG2* through the DR5 motif located in its distal promoter in human hepatocytes and that the motif prefers hCAR to pregnane X receptor.

Introduction

ABCG2 (also known as breast cancer resistance protein or BCRP) is a membrane-bound efflux transporter belonging to the ATP-binding cassette (ABC) superfamily and functions as a homodimer. It is expressed in various tissues including liver, intestine, brain, kidney and placenta [1,2], playing key roles in detoxification of xenobiotics [3,4]. Its substrates include endobiotics such as estrone sulfate [5] and urate [6], anticancer agents such as mitoxantrone, topotecan and methotrexate [7–10], and metabolites of environmental chemicals such as benzo[*a*]pyrene-3-sulfate and benzo[*a*]-pyrene-3-glucuronide [11]. ABCG2 is also known to be highly expressed in a subpopulation of stem cells from various sources and thus its important functions in these cells, such as the maintenance of the stem cell homeostasis against numerous stress and the protection from cell death, are suggested [12]. Recent studies also suggest critical roles of ABCG2 in cancer stem cells (CSCs) [13–18]. These cells have extensive proliferation and self-renewal capacity, which is associated with the acquisition of multidrug resistance in chemotherapy and the cancer recurrence [13–18]. ABCG2 is thus associated with not only the pharmacokinetics of drugs but the efficacy of chemotherapy to cure cancer as well.

The involvement of a couple of nuclear receptors (NRs) and other transcription factors in the xenobiotic- and endobiotic-induced expression of *ABCG2* has been reported. An estrogen receptor response element is located between −188 and −177 [19]. Peroxisome proliferator-activated receptor γ up-regulates the expression of *ABCG2* through the binding to three DR1 (direct repeat 1) motifs located between −3946 and −3796 [20]. Two aryl hydrocarbon receptor responsive elements have been found in the proximal (−194 to −190) and distal (−2357 to −2333) regions of the promoter [21,22]. Moreover, the expression of *ABCG2* is increased by the exposure to xenobiotics that do not interact with the above-mentioned transcription factors [23,24].

Constitutive androstane receptor (CAR) and pregnane X receptor (PXR) share their roles in the drug-induced expression of phase

Abbreviations: ABC, ATP-binding cassette; AdhCAR, hCAR-expressing adenovirus; BCRP, breast cancer resistance protein; CAR, constitutive androstane receptor; ChIP, chromatin immunoprecipitation; CITCO, 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldeyde *O*-(3,4-dichlorobenzyl)oxime; CSC, cancer stem cell; DMSO, dimethyl sulfoxide; h, human; hCAR-V5, hCAR-tagged with the V5 epitope at C-terminus; DR, direct repeat; EMSA, electrophoretic mobility shift assay; Luc, luciferase; MRP, multidrug resistance-associated protein; NR, nuclear receptor; PXR, pregnane X receptor; RXRα, retinoid X receptor α; TESS, transcriptional element search system.

* Corresponding author. Fax: +81 22 795 6826.
E-mail address: yoshinari@m.tohoku.ac.jp (K. Yoshinari).

0003-9861/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2011.10.014

I and phase II enzymes, and drug transporters in liver and other excretory tissues [25]. CAR is normally retained in cytoplasm and translocated into nucleus upon exposure to several drugs such as phenobarbital [26]. After the translocation to nucleus, CAR binds to *cis*-elements in the promoter region of target genes as a heterodimer with retinoid X receptor α (RXRα), and enhances their transcription [26]. Recent studies have reported that *ABCG2* mRNA levels in cultured human hepatocytes are increased after treatment with phenobarbital or a PXR activator rifampicin [23]. In addition, treatment of epileptic patients with carbamazepine, another human CAR (hCAR) activator, caused the increase in *ABCG2* mRNA levels in the liver [24]. These data strongly suggest that the activation of CAR enhances the transcription of *ABCG2* in human liver. In the present study, we have investigated the molecular mechanism for the CAR-dependent transcriptional activation of human *ABCG2* (*hABCG2*).

Materials and methods

Materials

T4 polynucleotide kinase was purchased from New England Biolabs (Ipswich, MA). Restriction enzymes were from New England Biolabs and TaKaRa Shuzo (Ohtsu, Japan). Poly(dI-dC) and [γ-^{32}P]ATP were purchased from GE Healthcare (Piscataway, NJ) and PerkinElmer (Waltham, MA), respectively. William's medium E, media supplements and fetal bovine serum were obtained form Invitrogen (Carlsbad, CA) and Nichirei biosciences (Tokyo, Japan), respectively. 6-(4-Chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldeyde *O*-(3,4-dichlorobenzyl)oxime (CITCO) and rifampicin were obtained from Merck (Darmstadt, Germany) and Sigma–Aldrich (St. Louis, MO), respectively. Matrigel and ITS-PREMIX were from BD-Biosciences (Heidelberg, Germany). KHEM5310 medium was from KAC (Kyoto, Japan). Oligonucleotides were synthesized by Fasmac (Atsugi, Japan). All other reagents were of highest grade available from Wako Pure Chemical Industries (Osaka, Japan) or Sigma–Aldrich.

Reporter gene constructs and expression plasmids

DNA fragments of *hABCG2* promoters were amplified with KOD-FX (Toyobo, Tokyo, Japan) and primers shown in Supplementary Table 1, and were subcloned into pGL4.10 (Promega, Madison, WI) to obtain a series of luciferase reporter constructs. Mutated constructs were prepared using QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) with a pair of primers 5′-ATCTGCATGTGACCCCTCCATTTAATTTCTTTCTCTCTGTCTATTC-3′ and 5′-GAATAGACAGAGAGAAAGAAATTAAATGGAGGGGTCACATG CAGAT-3′. Double-stranded oligonucleotides shown in Supplementary Table 2 were ligated into pGL3-Basic (Promega) having thymidine kinase promoter [27] to obtain the constructs containing three copies of wild-type, mutated and complement nuclear receptor binding motifs. $(NR1)_5$-tk-pGL3 and p3A4-362-7.7k were reported previously [27,28]. Expression plasmid for human PXR (pTargeT-hPXR) was constructed as follows: The PXR cDNA fragment was prepared from pTNT-hPXR [29] by digesting with XhoI and NotI, and was subcloned into the same restriction sites of empty pTargeT. Expression plasmid for hCAR (pTargeT-hCAR and pcDNA3.1-hCAR) and pTNT-based plasmids for in vitro synthesis of hCAR and hRXRα were prepared previously [28,29].

Reporter assay

HepG2 and MCF7 cells (RIKEN BioResource Center, Tsukuba, Japan) were cultured in Dulbecco's modified Eagle's medium (Wako Pure Chemicals) supplemented with 10% heat-inactivated fetal bovine serum, minimum essential medium nonessential amino acids and antibiotic–antimycotic. Cells were seeded in 24- or 48-well plate (BD Biosciences) at 1×10^5 or 3×10^4 cells/well, respectively, 24 h before transfection. Reporter construct and expression plasmid were cotransfected using Cellphect Transfection kit (GE Healthcare). phRL-TK Control Vector (Promega) was also cotransfected to normalize transfection efficiency. Twelve hours after transfection, the cells were treated with 0.5 μM CITCO or vehicle (0.1% dimethyl sulfoxide, DMSO) for 48 h.

Cryopreserved human hepatocytes (lot. HEP187111: white, female, 56-year-old), purchased from BIOPREDIC International (Rennes, France), were thawed and cultured using Hepatocytes isolation Kit (Xenotech, Lenexa, KS) according to the manufacturer's protocol. The cells were plated onto collagen-coated 48-well plate (BD Biosciences) at a density of 1×10^5 cells/well and maintained in KHEM5310 medium (KAC) supplemented with 10% fetal bovine serum for 4 h in an atmosphere of 5% CO_2/95% air at 37 °C. The medium was then changed to serum-free Williams' medium E containing 0.1 μM dexamethasone, ITS-PREMIX, 100 U/ml penicillin, 100 μg/ml streptomycin, and the cells were cultured for 24 h. Transfection was performed using jetPEI-Hepatocyte (PolyPlus Transfection, Illkirch, France) following the manufacturer's instruction. Transfected cells were treated with 0.5 μM CITCO or vehicle (0.1% DMSO) for 24 h.

Cell lysates were prepared with Passive Lysis Buffer (Promega) for luciferase assays using Dual-luciferase Reporter Assay System (Promega). The activities of firefly luciferase were normalized with those of *Renilla* luciferase.

Measurement of mRNA levels in human hepatocytes

Total RNA was prepared from cultured human hepatocytes that were infected with hCAR-expressing adenovirus or control virus in our previous study [29]. mRNA levels of *hABCG2* and *β-actin* were determined as described previously [29,30] using following primers; 5′-AAACTTCTGCCCAGGACTCAATG-3′ and 5′-GCCCTGCTTTAC-CAAATATTCTTCG-3′ for *hABCG2*, and 5′-ACCCTGTGCTGCTCACC GA-3′ and 5′-CTGGATGGCTACGTACATGGCT-3′ for *β-actin*.

Electrophoretic mobility shift assays (EMSAs)

hCAR and hRXRα were synthesized in vitro with pTNT-hCAR and pTNT-hRXRα, respectively, using TNT SP6 Quick Coupled Transcription/Translation System (Promega). The sequence of oligonucleotides used are shown in Fig. 3A. EMSAs were performed as described previously [28,29].

Establishment of HepG2 cell line stably expressing hCAR (hCi-19)

HepG2 cells were seeded in 10-cm culture dishes at 1×10^6 cells/dish a day before transfection, and pcDNA3.1-hCAR including the neomycin resistant gene was transfected using calcium phosphate method. Twelve hours after transfection, medium was changed. After 2-day culture, the cells were selected with 800 μg/ml G418 (Nacalai tesque, Kyoto, Japan). Medium was changed three times a week for 4 weeks until small colonies were visible. Twenty-one colonies were isolated and cultured in a large scale. In the present study, one of them (clone #19) was chosen because functional hCAR expression was relatively high based on the results with reverse transcription-PCR, Western blot analysis and luciferase reporter assays. This clone was named hCi-19. Characterization of these stable clones will be reported elsewhere (Imai et al., in preparation).