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ScienceDirect
Neuroscience Research 60 (2008) 307–313
Neuroscience Research
www.elsevier.com/locate/neures

Identification of negative transcriptional factor E4BP4-binding site in the mouse circadian-regulated gene *Mdr2*

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Received 8 August 2007; accepted 27 November 2007
Available online 23 December 2007

Abstract

The hepatic transporter *Mdr2* is an ATP-binding cassette transporter which excretes phosphatidylcholine into the bile. We showed that the level of *Mdr2* mRNA oscillated in circadian fashion in mouse liver whereas such oscillation was dampened in the liver of *Clock* mutants. To examine transcriptional regulation of the *Mdr2* gene we performed luciferase reporter assays using plasmid constructs containing the 5′-flanking region of the *Mdr2* gene. Reporter assays using deletion constructs demonstrated that E4BP4 represses the transcriptional activity of the promoter including the D1 and D2 sites within four putative E4BP4-binding sites. Chromatin immunoprecipitation and gel shift assays showed that E4BP4 binds to the D2 site, but not to the D1 site. These data suggested that E4BP4 is a negative transcription factor for circadian *Mdr2* mRNA expression.

Keywords: Circadian; Multidrug resistance 2 (Mdr2); ATP-binding cassette (ABC) transporter; E4BP4; Negative transcription; *cis*-Element

1. Introduction

P-glycoproteins were discovered as large cell membrane proteins that are overproduced in multidrug resistant (MDR) cancer cells (Juliano and Ling, 1976). P-glycoproteins are encoded by three highly homologous *Mdr* genes in rodents, *Mdr1* (*Mdr1b*), *Mdr2*, and, *Mdr3* (*Mdr1a*), and by two in humans, namely *MDR1* and *MDR3* (also called *MDR2*) (Gros et al., 1986, 1988; Chen et al., 1986; Ueda et al., 1987; Devault and Gros, 1990). Transfection studies have shown that P-glycoprotein can be separated into two groups. One includes Mdr1 and Mdr3, and human MDR1 can directly confer drug resistance upon drug-sensitive cells (Ueda et al., 1987; Devault and Gros, 1990). Whereas the other includes mouse Mdr2 and human MDR3 that cannot confer such resistance (Ueda et al., 1987; Devault and Gros, 1990; Schinkel et al., 1991; Gros et al., 1988). The function of *Mdr2* P-glycoprotein was discovered in mice that are homozygous for a disrupted *Mdr2* gene, which results in the absence of *Mdr2* P-glycoprotein from the canalicular membrane (Smit et al., 1993). $Mdr2^{-/-}$ mice do not excrete phospholipid into the bile, which contains very low levels of cholesterol. The absence of biliary phospholipid in $Mdr2^{-/-}$ could thus result in bile duct damage induced by toxic bile acids, ultimately leading to sclerosing cholangitis. Consequently, Smit et al. suggested that the Mdr2 P-glycoprotein acts as a flippase for major phospholipid in normal bile by transferring phosphatidylcholine (PC) molecules from the inner to the outer leaflet of the canalicular membrane. One study has indicated that bile flow and the biliary excretion of individual lipids and proteins are subject to circadian rhythms (Nakano et al., 1990).

Circadian rhythms in physiology and behavior in various organisms ranging from bacteria to humans are driven by endogenous oscillators called circadian clocks (Dunlap, 1999). Recent molecular and genomic analyses of clock genes have resulted in a generalized model based on the transcriptional/translational feedback loops of clock genes (Ishida et al., 2001; Panda et al., 2002; Reppert and Weaver, 2002). The positive

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0168-0102/$ – see front matter
doi:10.1016/j.neures.2007.11.014

element of the feedback loop is thought to consist of CLOCK and BMAL1 dimers, whereas several negative components comprise three *Period* (*mPer1*, *mPer2* and *mPer3*) (Shearman et al., 2000; Bae et al., 2001) and two *Cryptochrome* genes (*mCry1* and *mCry2*) (Kume et al., 1999; van der Horst et al., 1999; Griffin et al., 1999). Transcription of at least *mPer1* and probably *mCry1 mCry2* is activated by CLOCK/BMAL1 heterodimer binding to E-box elements in their gene promoters (Gekakis et al., 1998; Darlington et al., 1998). Once inside the nucleus, mPer stimulates *Bmal1* transcription, while mCRY1 and mCRY2 negatively regulate *mPer* and *mCry* transcription by abrogating CLOCK/BMAL1 activation (Kume et al., 1999).

The PAR family of bZIP factors in vertebrates comprises three polypeptides, D-box-binding protein (*DBP*), hepatic leukemia factor (*HLF*) and thyrotrophic embryonic factor (*TEF*) which is also known as vitellogenin gene binding protein (*VBP*) (Drolet et al., 1991; Hunger et al., 1992, 1996; Hass et al., 1995). The PAR factors are characterized by a high amino acid sequence similarity over their DNA-binding domains ([G/A]TTACGTAA[C/T]). Several other polypeptides have been identified with similar characteristics to those of the PAR family. The mammalian transcription factor E4 binding protein 4 (*E4BP4*) (Cowell et al., 1992) (also known as *NFIL3*) (Ikushima et al., 1997), *Caenorhabditis elegans* cell death selector (*CES-2*) (Metzstein et al., 1996) and the *Drosophila vrille* protein (George and Terracol, 1997) have very similar DNA-binding profiles to the PAR factor. In general, E4BP4 binds to the target sequence ([G/A]T[G/T]A[C/T]GTAA[C/T]) as a dimer (Cowell et al., 1992) and represses transcription by forming complexes with the TBP-binding protein Dr1 through the repression domain of E4BP4 (Cowell and Hurst, 1994, 1996). While E4BP4 is a repressor, the PAR factors have been characterized as transcriptional activators (Li and Hunger, 2001). In our previous data E4BP4 has been shown as a negative regulator for circadian expression of *Period2* gene (Ohno et al., 2007a,b). Furthermore, E4BP4 was shown to interact with PER2 through the carboxyl-terminal region containing the repression domain of E4BP4 and with CRY2 suggesting E4BP4 might be a component of the negative regulator complex of mammalian circadian clocks (Ohno et al., 2007a,b).

Recent microarray studies showed that many genes are significantly rhythmic in liver (Ueda et al., 2002; Oishi et al., 2003, 2005; Lowrey and Takahashi, 2004; Ishida, 2007). The liver is the largest solid organ of the body and comprises the major site of intermediate metabolism including the synthesis and degradation of cholesterol. Some cytochrome p450 enzymes involved in cholesterol synthesis such as cholesterol 7α-hydroxylase (*Cyp7a1*) and sterol 12α-hydroxylase (*Cyp8b1*) show circadian oscillation in the mouse liver (Ueda et al., 2002; Oishi et al., 2005). Here, we measured hepatic levels of *Mdr2* mRNA during a day and found day–night rhythmic expression. To investigate circadian related transcription factors affect *Mdr2* transcription, we analyzed the *Mdr2* promoter using luciferase reporter assays *in vitro*. We evaluated the transcription factors PAR-bZIP family binds to DNA-binding sites in the *Mdr2* promoter *in vivo*. Our results indicated that E4BP4 is a negative regulatory factor of *Mdr2* expression.

2. Materials and methods

2.1. Cell culture

Human and mouse hepatoma HepG2 and Hepa1-6 cells, respectively, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and a mixture of penicillin and streptomycin at 37 °C under a humidified 5% CO_2 atmosphere.

2.2. Northern blotting

Total RNA was prepared using ISOGEN (Nippon Gene) and then poly(A)$^+$ RNA was purified using a GenElute mRNA Miniprep Kit (Sigma–Aldrich). Northern blotting proceeded as described (Oishi et al., 2005). Probes labeled with ^{32}P were generated from cDNA fragments of *Mdr2* (bases 2208–2787; GenBank accession number, NM 008830).

2.3. Construction of Mdr2 promoter luciferase plasmids

The promoter fragment (−2567 to +357) was cloned in front of the luciferase expression vector pGL3-Basic (Promega) and designated pGL3-2567. A series of deletion constructs was produced using the restriction sites in the promoter (Fig. 2B).

2.4. Luciferase assay

The internal control comprised reporter plasmids containing the *Mdr2* promoter region or its derivatives co-transfected with pRL-SV40 (Promega) into HepG2 cells. Luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) and a Luminometer Model TD-20/20 (Turner Designs). The transcriptional activities were normalized relative to *Renilla* luciferase activities.

2.5. Gel shift assay

Gel shifts were examined as described (Onishi and Kiyama, 2003). Nucleotide sequences of 16-bp oligonucleotide probes for D1 and D2 sites were 5′-ATCAGGATGTAAAATG-3′ and 5′-CCAAATATGTAACTAC-3′, respectively. These probes were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (New England BioLabs). The probes were suspended in 10 μl of 16 mM HEPES (pH 7.5), 150 mM KCl, 16% (v/v) glycerol, 1.6 mM $MgCl_2$, 0.8 mM dithiothreitol, 0.4 mM PMSF, 1 mM EDTA, 0.8 mg/ml BSA, 0.06 mg/ml poly(dI–dC) and 0.01% NP40 in the presence of or absence of competitor oligonucleotide and incubated with E4BP4 protein. The samples were resolved by electrophoresis on 6% polyacrylamide gels in 40 mM Tris–acetate, 1 mM EDTA and 5% glycerol at 100 V for 1 h.

2.6. Chromatin immunoprecipitation (ChIP) assay

ChIP assays proceeded as described (Onishi and Kiyama, 2003). Hepa1-6 cells were crosslinked with 1% formaldehyde for 15 min at room temperature and then washed three times with ice-cold PBS. The cells were suspended in lysis buffer (25 mM Tris–HCl (pH 8.0), 140 mM NaCl, 1% Triton X-100, 0.1% SDS, 3 mM EDTA and 1 mM PMSF) on ice for 30 min. Sonication to shear DNA into 100–500 bp fragments was followed by centrifugation and supernatants containing soluble chromatin were collected. The chromatin fraction was incubated with anti-Myc antibody overnight at 4 °C, then with *Escherichia coli* DNA /Protein A/G PLUS-Agarose (Santa Cruz Biotechnology). Chromatin immunocomplexes were washed three times, once with wash buffer 1 (20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% Triton X-100 and 2 mM EDTA), once with buffer 2 (20 mM Tris–HCl (pH 8.0), 500 mM NaCl, 0.1% SDS, 1% Triton X-100 and 2 mM EDTA), and once with wash buffer 3 (10 mM Tris–HCl (pH 8.0), 0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate and 1 mM