Gene 546 (2014) 50–55

Contents lists available at ScienceDirect

Gene

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

Hepcidin expression in liver cells: evaluation of mRNA levels and transcriptional regulation

Yohei Kanamori [a], Masaru Murakami [b], Tohru Matsui [a], Masayuki Funaba [a,*]

[a] Division of Applied Biosciences, Kyoto University Graduate School of Agriculture, Kyoto 606-8502, Japan
[b] Laboratory of Molecular Biology, Azabu University School of Veterinary Medicine, Sagamihara 252-5201, Japan

ARTICLE INFO

Article history:
Received 3 February 2014
Received in revised form 7 May 2014
Accepted 19 May 2014
Available online 20 May 2014

Keywords:
Hepcidin
mRNA
Transcription
BMP
Liver cells

ABSTRACT

Hepcidin produced in the liver negatively regulates intestinal iron absorption, and the bone morphogenetic protein (BMP) pathway is well-known to stimulate hepcidin expression. However, the regulation of hepcidin expression has not been fully elucidated. In this study, we evaluate different systems that can be used to determine how hepcidin expression is regulated. The basal expression of hepcidin in liver cell lines, such as HepG2 cells and Hepa1-6 cells, was lower than that in the liver and primary hepatocytes; the expression levels of hepcidin in the cell lines were near the limit of detection for RT-PCR and RT-qPCR analyses. Treatment with trichostatin A, RNAlater, or MG-132 enhanced the expression of hepcidin in HepG2 cells, suggesting that histone deacetylation, instability of mRNA, or proteosomal degradation of the protein(s) that positively regulate hepcidin expression may be responsible for the decreased expression of hepcidin in HepG2 cells. In luciferase-based reporter assays, BMP induced the transcription of a reporter, hepcidin(−2018)-luc, that contains nt −2018 through nt −35 of the hepcidin promoter in HepG2 cells and Hepa1-6 cells. However, BRE-luc, a representative reporter used to evaluate BMP signaling, was unresponsive to BMP in HepG2 cells. These results suggest that hepcidin transcription can be best evaluated in liver cell lines and that the hepcidin promoter senses BMP signaling with high sensitivity. The present study demonstrates that studies regarding the regulation of hepcidin expression at the mRNA level should be evaluated in primary hepatocytes, and liver cell lines are well-suited for studies examining the transcriptional regulation of hepcidin.

1. Introduction

The iron uptake of the body is tightly regulated through the modulation of its absorption from the intestine (Lee and Beutler, 2009). Ferric iron is reduced at the luminal site by intestinal cytochrome b, and ferrous iron is transferred into the enterocyte via the transmembrane protein divalent metal transporter-1. On the basolateral side, ferrous iron is exported from enterocytes to the circulatory system via ferroportin, and after being oxidized by the membrane bound ferroxidase hephaestin, iron is incorporated into transferrin (Ganz and Nemeth, 2012).

Hepcidin is a 25-amino acid antimicrobial peptide that serves as the central regulator of intestinal iron absorption (Lee and Beutler, 2009). Hepcidin binds to the iron exporter ferroportin and induces its endocytosis and proteolysis, thus preventing intestinal absorption of iron (De Domenico et al., 2007; Nemeth et al., 2004). Previous studies suggested that hepcidin activity is mainly regulated at the gene transcript level (Ganz and Nemeth, 2012). The ratio of intestinal iron absorption to iron intake decreases as dietary iron levels increase (Laftah et al., 2004), and this is achieved by the up-regulation of hepatic hepcidin expression (Corradini et al., 2009; Kautz et al., 2008). The current model suggests that hepcidin expression is transcriptionally regulated by the pathway induced by bone morphogenetic proteins (BMPs) such as BMP2, BMP4, BMP6 and BMP9 (Babitt et al., 2006, 2007; Lee and Beutler, 2009; Muckenthaler, 2008; Truksa et al., 2006). Increased levels of iron have been shown to increase the hepatic expression of BMP6 (Kautz et al., 2008), and the disruption of BMP6 resulted in decreased hepatic hepcidin expression and the accumulation of iron in the liver (Andriopoulos et al., 2009; Meynard et al., 2009). However, factors other than the BMPs are also involved in modulating hepcidin expression (Goodnough et al., 2012; Lee and Beutler, 2009; Muckenthaler, 2008), indicating that the mechanisms governing hepcidin expression have not yet been fully elucidated.

Abbreviations: BMP, bone morphogenetic protein; CMF, calcium and magnesium-free; HBSS, Hank's buffered salt solution; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; ALK, activin receptor-like kinase; RT-PCR, reverse transcription-polymerase chain reaction; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; pCMV-βGal, plasmid expressing β-galactosidase under the control cytomegalovirus promoter; SEM, standard error of the mean; Id1, inhibitor of differentiation 1; Hprt1, hypoxanthine phosphoribosyltransferase 1.

* Corresponding author at: Division of Applied Biosciences, Kyoto University Graduate School of Agriculture, Kitashirakawa Oiwakecho, Kyoto 606-8502, Japan.
E-mail address: mfunaba@kais.kyoto-u.ac.jp (M. Funaba).

http://dx.doi.org/10.1016/j.gene.2014.05.040
0378-1119/

While exploring the factors that affect hepcidin expression, we noticed that hepcidin expression in liver cell lines is extremely low compared to that in the liver and primary hepatocytes, and this low level of expression makes it difficult to evaluate the regulation of hepcidin at the mRNA level. The main objective of this study is to clarify the differences of the hepcidin expression level among the livers, primary hepatocytes and liver cell lines. The present study provides information on cultured cell systems suitable for evaluating hepcidin expression.

2. Materials & methods

2.1. Animals and cell culture

Animal experiments were approved by the Kyoto University Animal Experiment Committee or the Azabu University Animal Experiment Committee. Mouse and rat livers were obtained from normal adult C57BL/6 mice and Sprague–Dawley rats, respectively. Primary rat hepatocytes were isolated by collagenase digestion of livers from male Wistar rats weighing 200 to 300 g. Livers were perfused from the portal vein to the incised inferior vena cava with calcium and magnesium-free (CMF) buffer consisting of 40 mM Hepes, pH 7.4, 120 mM NaCl, 5.4 mM KCl, 5.0 mM $NaHCO_3$ and 5.6 mM glucose supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 250 ng/mL amphotericin B for 10 min at a rate of ~12 mL/min followed by perfusion with CMF buffer containing 0.05% collagenase (Wako, Tokyo, Japan) for 10 min. Subsequently, hepatocytes were liberated into Hank's buffered salt solution (HBSS), i.e., 140 mM NaCl, 5.4 mM KCl, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 0.81 mM $MgSO_4 \cdot 7H_2O$, 1.3 mM $CaCl_2$, 4.2 mM $NaHCO_3$ and 5.6 mM glucose supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 250 ng/mL amphotericin B. After cell recovery by centrifugation at 50 ×*g* for 2 min, the cells were washed with HBSS three times and resuspended in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and antibiotics. Cells (>90% hepatocytes by microscopy) were seeded on collagen-coated plates at 1.5×10^5 cells per well in 12-well plates. After attachment, the medium was replaced with the same medium, and the cells were incubated for 24 h. Then, the cells were treated with or without BMP2 (4 nM; R&D Systems, Minneapolis, MN, USA) in the presence or absence of cycloheximide (0.5 μg/mL) for 12 h.

HepG2 hepatoma cells, Hepa1-6 hepatoma cells and 3T3-L1 preadipocytes were cultured in DMEM with 10% FBS and antibiotics. For the luciferase-based reporter assays, these cells were transiently transfected using PolyFect transfection reagent (Qiagen, Valencia, CA, USA) or polyethylenimine Max reagent (Polysciences, Warrington, PA, USA), according to the manufacturers' protocols. At 24 h post-transfection, the cells were pre-treated with or without LDN-193189 (100 nM), an inhibitor of the BMP type I receptor (Cuny et al., 2008), for 15 min before BMP2 (4 nM) treatment for 16 h. To evaluate the expression of constitutively active activin receptor-like kinase (ALK) 3 (ALK3(QD)), cells were harvested at 36 h post-transfection. To evaluate the levels of hepcidin mRNA, HepG2 cells and Hepa1-6 cells were treated for 24 h with or without trichostatin A (TSA: 0.1 μg/mL; Wako, Tokyo, Japan), an inhibitor of class I and II histone deacetylases (Gräff and Tsai, 2013), RNAlater (0.1 or 1%; Life Technologies, Carlsbad, CA, USA) to denature RNase at a controlled pH (Zaitoun et al., 2010) or MG-132 (20 μM; EMD Millipore, Billerica, MA, USA), a proteasome inhibitor (Tsubuki et al., 1993). In Hepa1-6 cells, treatment with MG-132 at 20 μM caused detachment of the cells from culture dish, and, therefore, we also treated with MG-132 at 10 μM.

2.2. RNA isolation, RT-PCR and RT-quantitative PCR

Total RNA was isolated from the livers and cells, and cDNA was synthesized using TRIZOL (Invitrogen, Grand Island, NY, USA) and ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan), respectively, according to the manufacturers' protocols. The cDNA reverse-transcribed from 5 or 20 ng of total RNA was used as a template for conventional reverse transcription-polymerase chain reaction (RT-PCR) or RT-quantitative PCR (RT-qPCR) using SYBR Green I (Thunderbird SYBR qPCR mix, Toyobo, Osaka, Japan) as described previously (Asano et al., 2013). The oligonucleotide primers for the conventional RT-PCR and RT-qPCR are presented in Table 1. The Ct value was determined, and the abundance of gene transcripts was analyzed using the $^{\Delta\Delta}$Ct method using glyceraldehyde-3-phosphate dehydrogenase (Gapdh) as the normalization gene (Duran et al., 2005).

2.3. Plasmids and reporter assays

Plasmids were obtained as follows: BRE-luc reporter plasmids (Korchynskyi and ten Dijke, 2002) was from Dr. P. ten Dijke, and ALK3(QD) (Imamura et al., 1997) was obtained from Dr. K. Miyazono. The DNA fragment spanning from −2018 bp to −35 bp of the mouse hepcidin (Hamp1) promoter was amplified and cloned into the basic vector pGL4 containing the firefly luciferase reporter (Hepcidin (−2018)-luc) with nt +1 as the translation initiation site. The product was verified through nucleotide sequencing. Luciferase-based reporter

Table 1
Oligonucleotide PCR primers for RT-PCR and RT-qPCR.

	Oligonucleotide		GenBank accession number
	5′-primer	3′-primer	
RT-PCR and qPCR:			
Gapdh	5′-TTCATTGACCTCAACTACATGGT-3′	5′-GCTAAGCAGTTGGTGGTGCAGGA-3′	NM_002046 (human) NM_008084 (mouse) NM_017008 (rat)
Hprt1	5′-ATGGGAGGCCATCACATTG-3′	5′-CTTTCCAGTTAAAGTTGAGAGATCA-3′	NM_000194 (human) NM_013556 (mouse) NM_012583 (rat)
mhHepcidin[a]	5′-TGCCTCCTGCTCCTCCTCCT-3′	5′-GCAGAAAATGCAGATGGGGAAGT-3′	NM_021175 (human) NM_032541 (mouse)
mrHepcidin[b]	5′-GCTGCCTGTCTCCTGCTT-3′	5′-TTACAGCATTTACAGCAGAAGAGG-3′	NM_053469 (rat) NM_032541 (mouse)
RT-PCR:			
Tmprss6	5′-CACTGTGACTGTGGCCTCCAGG-3′	5′-CGTCGTAGTCATGGCTGTCCTC-3′	NM_153609 (human) NM_027902 (mouse) NM_001130556 (rat)
RT-qPCR:			
rGapdh	5′-ACAACTTTGGCATCGTGGA-3′	5′-CTTCTGAGTGGCAGTGATGG-3′	NM_017008 (rat)
rHepcidin	5′-GCTGCCTGTCTCCTGCTT-3′	5′-AGCCGTAGTCTGTCTCGTCT-3′	NM_053469 (rat)

[a] PCR primers common to mouse and human hepcidin.
[b] PCR primers common to mouse and rat hepcidin.