Biochimica et Biophysica Acta 1829 (2013) 1257–1265

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Regulation of HFE expression by poly(ADP-ribose) polymerase-1 (PARP1) through an inverted repeat DNA sequence in the distal promoter

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ARTICLE INFO

Article history:
Received 17 June 2013
Received in revised form 28 September 2013
Accepted 22 October 2013
Available online 29 October 2013

Keywords:
HFE
PARP1
Negative element
Inverted repeat
Cruciform
HAMP

ABSTRACT

Hereditary hemochromatosis (HH) is a common autosomal recessive disorder of iron overload among Caucasians of northern European descent. Over 85% of all cases with HH are due to mutations in the hemochromatosis protein (HFE) involved in iron metabolism. Although the importance in iron homeostasis is well recognized, the mechanism of sensing and regulating iron absorption by HFE, especially in the absence of iron response element in its gene, is not fully understood. In this report, we have identified an inverted repeat sequence (ATGGTcttACCTA) within 1700 bp (−1675/+35) of the HFE promoter capable to form cruciform structure that binds PARP1 and strongly represses HFE promoter. Knockdown of PARP1 increases HFE mRNA and protein. Similarly, hemin or $FeCl_3$ treatments resulted in increase in HFE expression by reducing nuclear PARP1 pool via its apoptosis induced cleavage, leading to upregulation of the iron regulatory hormone hepcidin mRNA. Thus, PARP1 binding to the inverted repeat sequence on the HFE promoter may serve as a novel iron sensing mechanism as increased iron level can trigger PARP1 cleavage and relief of HFE transcriptional repression.

1. Introduction

Hereditary hemochromatosis is (HH) an autosomal recessively inherited disorder of iron metabolism, affecting about 1 in 400 individuals of Northern European descent. The disease is characterized by excessive intestinal iron absorption and progressive iron overload throughout the body [reviewed in [1]], leading to damaged liver. Feder et al. [2] showed that homozygosity for mutation (C282Y, G > A) in the HFE gene was responsible for common phenotypic HH. This single mutation in the HFE accounts for approximately 85% of HH cases, although other rare mutations in the HFE coding region as well as mutations in few other genes involved in iron metabolism such as Transferrin Receptor 2, hepcidin, ferroportin, and hemojuvelin have also been associated with HH and abnormal iron profile [3,4]. Iron overload resulting from mutations of HFE or these genes occurs due to dysregulation of the iron-regulatory hormone, hepcidin encoded by HAMP gene, produced primarily in the liver. Hepcidin negatively regulates iron absorption and recycling by reducing surface levels of the iron export protein, ferroportin on duodenal enterocytes and macrophages [46].

The *Hfe* knockout ($Hfe^{-/-}$) and C282Y knock-in *Hfe* mice develop iron, ferroportin on duodenal enterocytes and macrophages [5–7]. HFE is an atypical member of the major histocompatibility complex class I proteins. Similar to other members of this family, HFE consists of a transmembrane domain and a short cytoplasmic tail [reviewed in [8]]. However, unlike typical members of the family, it does not contain the peptide binding domain and is not involved in antigen peptide-presentation. Rather studies from HFE deficient patients and Hfe-knockout mice with iron overload suggest a role in HAMP expression [47]. Thus, importance of HFE in iron regulation became apparent from these and other studies with HH patients and murine models, the underlying mechanism by which HFE regulates iron homeostasis is only beginning to be understood. The data obtained thus far strongly suggest that modifier genes contribute to regulatory capacity of the HFE gene [5] and a signaling pathway that senses iron status may depend on the HFE gene.

Poly(ADP-ribose) polymerase-1 (PARP1) is a ubiquitous, abundant and highly conserved nuclear protein of ~116 kDa [9]. It is the founding member of the PARP family and accounts for more than 90% of catalytic activity in cells [10]. PARP1 is a multifunctional protein and has a number of biochemical activities. It possesses an NAD^+-dependent catalytic activity that cleaves NAD^+ into nicotinamide and ADP-ribose, then polymerizes the latter into negatively charged polymer called poly(ADP-ribose) or PAR on target proteins. In addition, PARP has been shown to be involved in the regulation of chromatin structure and transcription, DNA methylation, insulator activity, and chromosome organization [reviewed in [11]].

PARP1 binds to DNA using an amino terminal, DNA binding domain with three zinc fingers, one of which is required for NAD^+-dependent

Abbreviations: HH, hemochromatosis; PARP1, poly(ADP-ribose) polymerase-1; HFE, hemochromatosis protein

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http://dx.doi.org/10.1016/j.bbagrm.2013.10.002

catalytic activity of PARP1. Its carboxyl-terminal catalytic domain catalyzes PARylation of target proteins [12,13]. Genome-wide search localized PARP1 to the promoters and enhancers of many actively transcribed genes, and the pattern correlates with active gene expression [14]. A cross-shaped DNA structure, called cruciform DNA, observable under the electron microscope, can be formed by complementary perfect or imperfect inverted repeats of 6 or more nucleotides in the DNA sequence. The sequence refolds into hairpin loops on opposite strand across from each other. Among the DNA binding proteins, PARP1 exhibits only a weak sequence preference but binds preferentially to cruciform structures. The order of PARP1's substrate preference has been shown to be: cruciform > loop > linear DNA [15]. PARP1 binding to cruciform structure in plasmid DNA results in relaxed plasmid DNA conformation [16].

Some studies on transcriptional regulation by PARP1 provided evidence that the enzymatic activity of PARP1 is required [17,18], whereas others have indicated that it is not [19–21]. Thus, transcriptional regulation by PARP1 may or may not require its enzymatic activity.

In this report, to gain new insight into human HFE expression, we focused on a 1700 bp HFE promoter including the transcription start site and have identified a negative element, an inverted repeat sequence, in the distal HFE promote. We further demonstrated that nuclear protein, PARP1 bound to this sequence negatively regulating HFE expression. Diminishing cellular PARP1 by means including iron treatment increases HFE. We speculate that, in the absence of iron response element, HFE utilizes PARP1 in its own expression in response to excess iron.

2. Materials and methods

2.1. Plasmid constructs

A 1674 bp human HFE promoter fragment (−1638 to +37) in the promoterless luciferase reporter vector pGL3-Basic (Promega) was PCR generated using primer pairs H1m and H4 (Table 1), digested and cloned at Sma I and Kpn I sites of the vector. Promoter deletion constructs, H870 bp and H210 bp, in pGL3-Basic were cloned using Sac I and Hind III sites of the vector, respectively. The sequential deletion constructs between H871 and H214 were created by PCR cloning using a forward primer harboring the designed Kpn I site in combination with the reverse primer GL3R in the vector (Table 1). Following PCR, the fragment of varying sizes was restricted and cloned into Kpn I site of the vector. The orientations of the inserts were determined by Hind III digestion. The sequences were verified by sequencing.

All PCRs were performed in similar fashion: following a denaturation step at 95 °C for 3 min, 30 cycles of 94 °C for 30 s, respective annealed temperature (53–60 °C) for 45 s, and 72 °C for 45 s, with a final extension of 7 min at 72 °C.

The putative repressor binding site in H810 was deleted using the primer pairs H72d1 and d2 (Table 1) and the QuikChange site-directed mutagenesis kit (Stratagene) following the supplier's instruction. The back slashes within the primer sequence represent the deletion site. Following verification by sequencing, the deleted fragment was subcloned into pGL3 vector. After 5′-phosphorylation, duplex H72d1 and d2 primers were cloned at Sma I site, 5′ to the H210 construct (dH210) as well as to the 200 bp proximal PKD1 promoter (dPKD). Wild-type duplex primers (hfee11 and 12) in the same region were also cloned at Sma I site, 5′ to the H210 construct (pH210) as well as to the 200 bp proximal PKD1 promoter (pPKD). Orientations of these constructs (d or p) were confirmed by PCR.

2.2. Cells, transfections and reporter assays

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose, HCT116 $p53^{-/-}$ cells were maintained in McCoy's media, HepG2 and HeLa in Eagle's minimal essential medium with 2 mM L-glutamate, and 1 mM pyruvate. All media are supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and antibiotic (100 IU/ml penicillin, 100 μg/ml streptomycin). All cells were grown at 37 °C supplied with 5% CO_2. Following overnight culturing in six-well (or 12-well) plates, the cells were transfected using either the calcium phosphate method (HEK293T and HCT116 cells) or with Lipofectamine (Gibco) (HepG2, HeLa cells) according to the manufacturer's instruction. A β-galactosidase expression plasmid (50 ng) was included to monitor transfection efficiency. After 6 h, the DNA-containing medium was removed, and the cells were incubated with growth media containing, and harvested at 40 h as described earlier [22]. Luciferase assays were carried out in 1× reporter lysis buffer (Promega) using Luminol

Table 1
Sequences of the primers used and their purposes.

Primers	Constructs	Sequence	Purpose
H1m	H1675	Forward:5′-tgcactccaacccgggcaatag-3′	PCR cloning of 1.7 kb promoter
H4		Reverse: 5′-ctctcctacagcagaaggtacc-3′	
GL3R		Reverse:5′-gctagcacgcgtaagagctcg-3′	Reverse primer for PCR cloning
H5	H555	5′-gatattttggtaccgactttatc-3′	Forward primers for PCR cloning
H7	H612	5′-gccaccttaggtaccttccacctg-3′	with GL3R at Kpn I site
H73	H810	5′-gtatctgggtaccaggatgatg-3′	
H72	H745	5′-gtgcatggtggtacctataatttg-3′	
H71	H670	5′-ctattataatggtaccatgatgaacttgggg-3′	
H72.3	H795	5′-gctatacaaggtaccattaaactgtgc-3′	
H72.2	H770	5′-caatctaaaggtaccctgtgcatggtc-3′	
H72.1	H751	5′-ctaaaattaaacggtacctggtcttacc-3′	
H72d1	HΔ810, ΔH210, ΔPKD	5′-caatctaaaattaaactgtg/aatttgttaagaaaagc-3′	Deletion, cloning
H72d2		5′-gcttttcttaacaaatt/cacagtttaattttagattg-3′	
hfee11	pH210, pPKD	5′-caatctaaaattaaactgtgcatggtcttacctat-3′	EMSA, pull-down, cloning
hfee12		5′- ataggtaagaccatgcacagtttaattttagattg-3′	
hfee14		5′-actgtgcatggtcttacctat-3′	EMSA, pull-down
hfee15		5′-ataggtaagaccatgcacagt-3′	
h63df		Forward: 5′ctcagagcaggaccttggtc-3′	HFE mRNA
hh4r		Reverse: 5′-ataccgtacttccagtagccct-3′	
L7-1		Forward: 5′-gcttcgaaaggcaaggaggaagc-3′	Ribosomal L7 mRNA- control
L7-2		Reverse:5′-tcctccatgcagatgatg-3′	
Hpcdn3		Forward:5′-catgttccagaggccaag-3′	Hepcidin mRNA
Hpcdn2		Reverse:5′caagacctgaattctggggcagc-3′	
H7r		Reverse:5′-cctgtcttccaagttcacc-5′	ChIP
Hfee1	With H7r	Forward: 5′-gagtgacaggatgatgttatttga-3′	ChIP endogenous
GL3F	With H7r	Forward: 5′-ctagcaaaataggctgtccc-3′	ChIP exogenous