Biochimica et Biophysica Acta 1829 (2013) 905–915

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

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

Starvation actively inhibits splicing of glucose-6-phosphate dehydrogenase mRNA *via* a bifunctional ESE/ESS element bound by hnRNP K

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ARTICLE INFO

Article history:
Received 7 March 2013
Received in revised form 17 April 2013
Accepted 22 April 2013
Available online 28 April 2013

Keywords:
mRNA splicing
hnRNP K
SRSF3
Nutrient regulation
Liver

ABSTRACT

Regulated expression of glucose-6-phosphate dehydrogenase (G6PD) is due to changes in the rate of pre-mRNA splicing and not changes in its transcription. Starvation alters pre-mRNA splicing by decreasing the rate of intron removal, leading to intron retention and a decrease in the accumulation of mature mRNA. A regulatory element within exon 12 of G6PD pre-mRNA controls splicing efficiency. Starvation caused an increase in the expression of heterogeneous nuclear ribonucleoprotein (hnRNP) K protein and this increase coincided with the increase in the binding of hnRNP K to the regulatory element and a decrease in the expression of G6PD mRNA. HnRNP K bound to two C-rich motifs forming an ESS within exon 12. Overexpression of hnRNP K decreased the splicing and expression of G6PD mRNA, while siRNA-mediated depletion of hnRNP K caused an increase in the splicing and expression of G6PD mRNA. Binding of hnRNP K to the regulatory element was enhanced *in vivo* by starvation coinciding with a decrease in G6PD mRNA. HnRNP K binding to the C-rich motifs blocked binding of serine-arginine rich, splicing factor 3 (SRSF3), a splicing enhancer. Thus hnRNP K is a nutrient regulated splicing factor responsible for the inhibition of the splicing of G6PD during starvation.

1. Introduction

Maintenance of energy homeostasis throughout the body is achieved by balancing the use of dietary energy to meet immediate needs and the storage of excess energy for use in the absence of dietary intake. A key pathway involved in this process is lipogenesis, by which excess energy in the form of carbohydrate is converted to triacylglycerol for storage in adipose tissue and as such this pathway is highly active and regulated in adipose tissue and liver. The enzymes involved in lipogenesis, ATP-citrate lyase, malic enzyme, fatty acid synthase, glucose-6-phosphate dehydrogenase (G6PD),[2] and acetyl-CoA carboxylase increase in amount during consumption of high-carbohydrate diets in order to increase a cell's capacity for lipogenesis; however, in response to starvation the amount of these proteins is decreased [1]. Dietary regulation of these proteins is controlled by both dietary nutrients and by changes in the amounts of key regulatory hormones such as insulin and glucagon. Regulation of most of the lipogenic enzymes is primarily through changes in the transcriptional activity of their genes [1]. G6PD is an exception in this family. Nutrient and hormonal regulation of G6PD expression are exclusively by changes in the degree of splicing of its mRNA [2,3].

RNA splicing is an essential step in gene expression that joins together the exons in the nascent transcript [4]. Splicing is a highly regulated process such that a single transcript can generate multiple mature mRNA *via* a process called alternative splicing. Evidence from global RNA sequencing indicates that alternative splicing is widespread and crucial for the proper function and fate of a cell [5–8]. In addition to alternative exon inclusion, the recognition and splicing of constitutive exons that are always present in the mature mRNA is regulated. Regulation of constitutive exon splicing can result in retained introns, which can trigger degradation of the mRNA presumably through nonsense-mediated decay [9]. This latter category is the type of alternative splicing that regulates expression of G6PD [3]. Alternative splicing and intron retention share regulatory mechanisms that involve the binding of splicing regulatory proteins to cis-acting elements within the mRNA. Within exons, these sequences are called exonic splicing enhancers (ESE) or exonic splicing silencers (ESS). Two protein families that include serine-arginine rich (SR) proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs) are known to function as trans-acting factors, which regulate splicing through binding to the ESE and ESS elements, respectively [10–15]. As such, these proteins contain RNA recognition motifs by which they can bind to the pre-mRNA.

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[2] CMV, cytomegalovirus; EMSA, electrophoretic mobility shift assay; ESE, exonic splicing enhancer; ESS, exonic splicing silencer; G6PD, glucose-6-phosphate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; HEX™, hexachlorofluorescein; hnRNP, heterogeneous nuclear ribonucleoprotein; RFP, red fluorescent protein; RIP, RNA immunoprecipitation; SRSF3, serine-arginine rich, splicing factor 3.

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http://dx.doi.org/10.1016/j.bbagrm.2013.04.009

The mechanisms by which SR proteins and hnRNPs respond to environmental and cellular cues are only now emerging. Serine phosphorylation of SR proteins is a primary mechanism regulating their activity, and phosphorylation is associated with enhanced splicing of the SR protein targets [16]. In contrast, hnRNPs act both as enhancers or inhibitors of mRNA splicing as well as regulate other steps in gene expression [17–19]. Many hnRNPs can be phosphorylated, methylated on arginines and modified by sumoylation [20,21]. Thus, hnRNPs could be candidates for nutritional regulation of splicing.

The observation that nutritional status can regulate RNA splicing has widespread implications for regulation of cellular function. G6PD provides a useful model to study splicing changes that occur in response to nutritional status because this is the sole mechanism regulating the accumulation of its mRNA. In mouse liver, accumulation of spliced G6PD mRNA occurs following consumption of a high carbohydrate, low-fat diet. Conversely, the amount of mature mRNA is decreased significantly by starvation or consumption of a diet high in polyunsaturated fatty acids [3,22,23]. The decrease in splicing of the nascent transcript is associated with a decrease in the removal of introns surrounding exon 12 of the G6PD mRNA and G6PD pre-mRNA containing these retained introns accumulates prior to its degradation [23]. Exon 12 contains the regulatory elements that are necessary for the nutrient regulation of splicing, which include both an ESS and an ESE [3,24]. The SR protein, SR splicing factor 3 (SRSF3) binds to the ESE within exon 12, thus increasing splicing of G6PD pre-mRNA during refeeding [24]. The increase in splicing of G6PD pre-mRNA results in accumulation of mature mRNA and ultimately more G6PD enzyme activity. The question remains, is inefficient splicing of exon 12 the default state to which liver reverts during starvation? In addition to SRSF3, we identified 3 members of the hnRNP family of proteins, K, L and A2/B1 that bind to this element *in vitro* [25]. In the present report, we test the hypothesis that hnRNP K, L and/or A2/B1 inhibit splicing of the G6PD mRNA and are involved in the inhibition of G6PD expression during starvation. We found that expression of hnRNP K increased during starvation, which resulted in an increase in its binding to the ESS with exon 12. HnRNP K inhibited splicing of the G6PD nascent transcript as well as a splicing reporter that contains the exon 12 regulatory element. Conversely, siRNA-mediated depletion of hnRNP K resulted in increased splicing of the G6PD pre-mRNA. Finally, hnRNP K and SRSF3 bind to the same sequences within the regulatory element and do so in a mutually exclusive manner. We propose that hnRNP K is a nutrient-regulated silencer of RNA splicing.

2. Material and methods

All animal experiments were conducted in conformity with the Public Health Service policy on Human Care and Use of Laboratory Animals, additionally the Institutional Animal Care and Use Committee of the Division of Laboratory Animal Resources at West Virginia University approved all experimental procedures.

2.1. RNA electrophoretic mobility shift assay (EMSA)

The RNA EMSA protocol is a modification of existing methods [26,27]. Briefly, 100 fmol of a 5′-hexachlorofluorescein (HEX™) labelled-RNA probe (IDT) corresponding to nucleotides (nt) 50–84 of Exon 12 was mixed with 92 ng of purified recombinant FLAG-tagged hnRNP K (Origene) in 1× binding buffer (10 mM Tris pH 7.3, 1 mM $MgCl_2$, 20 mM KCl, 1 mM DTT, 1 U of SuperRNasin (Ambion)) plus/minus unlabeled competitor oligonucleotides (0, 1 pmol, 2.5 pmol, 5 pmol, 7.5 pmol, 10 pmol; IDT) in a total reaction volume of 20 μL. The reactions were incubated for 30 min at room temperature. Supershift reactions received 1 μg of anti-hnRNP K (3C2, Abcam) 20 min into the initial binding reaction and the reaction proceeded another 10 min. The samples were loaded onto a pre-running 5% native polyacrylamide gel. The gel was imaged directly on a Typhoon 9410 Imager and signals were quantified using ImageQuant TL software. RNA EMSAs involving competition between hnRNP K and SRSF3 were incubated in the conditions as previously described [24]. Purified recombinant FLAG-tagged hnRNP K (Origene) and purified recombinant glutathione S-transferase (GST) conjugated-SRSF3 (Abnova) were incubated in 1× binding buffer (10 mM Tris pH 7.5, 1 mM MgCl2, 100 mM KCl, 0.1 mM DTT, 5% glycerol; Ref. [25]) in a total reaction volume of 20 μL with 100 fmol of a 5′-hexachlorofluorescein (HEX™) labelled-RNA probe (IDT) corresponding to nt 50–84 of Exon 12. Reactions were incubated and analyzed as previously described [24].

2.2. Western analysis

Whole cell lysates [24] and nuclear extracts [25] were prepared as described. After gel electrophoresis, the proteins were transferred to PDVF membrane (Bio-Rad), and probed with the antibodies as indicated in the figure legends. HnRNP A2/B1, hnRNP L and hnRNP K specific antibodies (Santa Cruz Biotechnology) and β-tubulin antibody (Cell Signalling) were obtained from the indicated sources. Secondary antibodies were conjugated to horseradish peroxidase and the antibody interactions were detected using ECL plus (GE Healthcare) followed by visualization on film and a Typhoon 9410 Imager (GE Healthcare). Signals were quantified with ImageJ (NIH) and ImageQuant TL (Molecular Dynamics), respectively. To verify the accuracy of the protein quantitation in the nuclear extracts, the extracts were run on a polyacrylamide gel and silver stained. The overall intensity of the visualized bands was similar betweens starved and refed samples (data not shown).

2.3. RNA isolation and measurement

Total cellular RNA was isolated using TRI Reagent® (Molecular Research Center). The total RNA was then digested with DNase I (Turbo DNA-free, Invitrogen) according to the manufacture's protocol. Specific RNA amounts were quantified by RT-qPCR (ICYCLER, Bio-Rad) using the QuantiTect SYBR Green kit (Qiagen) and primers listed in Supplemental Table 1. All reactions were carried out in duplicate and annealing temperatures for each set of primers were determined by using melting curves. The amount of each mRNA was calculated using a relative standard curve. All RNA samples were also amplified in the absence of reverse transcriptase to test for DNA contamination. Samples with DNA contamination were digested with DNase I a second time.

2.4. HnRNP K overexpression and RNA reporters

HepG2 (human hepatoma) cells were generated that stably express the RNA splicing reporters: Exon 12(+) or Exon 12(−) (Fig. 2) and were used for experiments testing the effect of overexpressing hnRNP K. The Exon 12(+) reporter contains mouse genomic DNA comprising nt 38–93 of exon 12 through the end of the G6PD gene as departed by a repetitive element, ligated to β-galactosidase and expression was driven by the CMV promoter. The cells were co-transfected with a plasmid expressing the neomycin resistance gene. Stable transformants were selected with G418 and then clonally isolated so that all cells reflect a single site of insertion of the RNA reporter. The Exon 12(−) reporter was constructed as the Exon 12(+) reporter but contains only the last 8 nt of exon 12 to preserve the 5′ splice site of the intron. One day after plating 2×10^5 cells in a 6 well plate, HepG2 cells were transfected with 3 μg of an expression vector for hnRNP K (Origene) or a control plasmid that expressed red fluorescent protein (RFP; pEGFP-N1, Clontech with RFP substituted for GFP; gift of Peter Stoilov) using the Express-in transfection reagent (Thermo Scientific) in DMEM containing 10% FBS. Total RNA and whole-cell protein extracts were collected after 48 h. Cell lysates were prepared using RIPA buffer plus phosphatase and protease inhibitors (Thermo Scientific). The quantitation of spliced *versus* unspliced reporter RNA used RT-qPCR and primers that produced 218 bp and 119 bp products, respectively.