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Regulation of mitochondrial morphology and cell survival by Mitogenin I and mitochondrial single-stranded DNA binding protein

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Abstract

We found that a mouse homolog of human DNA polymerase delta interacting protein 38, referred to as Mitogenin I in this paper, and mitochondrial single-stranded DNA-binding protein (mtSSB), identified as upregulated genes in the heart of mice with juvenile visceral steatosis, play a role in the regulation of mitochondrial morphology. We demonstrated that overexpression of Mitogenin I or mtSSB increased elongated or fragmented mitochondria in mouse C2C12 myoblast cells, respectively. On the other hand, the silencing of Mitogenin I or mtSSB by RNA interference led to an increase in fragmented or elongated mitochondria in the cells, respectively, suggesting that Mitogenin I and mtSSB are involved in the processes of mitochondrial fusion and fission, respectively. In addition, we showed that the silencing of Mitogenin I resulted in an increase in the number of trypan blue-positive cells and the silencing of mtSSB resulted in an enhancement of the sensitivity of the cells to apoptotic stimulation by etoposide. The present results demonstrated that these proteins play a role in cell survival.

Keywords: JVS; mtSSB; PDIP38; Mitochondria; Fission/fusion; Mitochondrial biogenesis; Apoptosis

1. Introduction

Mice with juvenile visceral steatosis (JVS) exhibit cardiac hypertrophy, fatty liver, hyperammonemia, hypoglycemia, and growth retardation; these characteristics are inherited in an autosomal recessive manner [1–3]. In JVS mice, the system responsible for the reabsorption of carnitine in the kidney is defective [4], and, as a result, carnitine levels are low in the serum, liver, and skeletal and cardiac muscles [3,5,6]. It was previously shown that the primary systemic carnitine deficiency in JVS mice is caused by dysfunctional organic cation/carnitine transporter 2 (OCTN2) [7,8]. In addition to these pathological features, the cardiac and skeletal cells of JVS mice exhibit a markedly increased number of mitochondria [5,6]. Mitochondrial morphology was also shown to be abnormal in the cardiac cells of these mice [6]. However, the mechanisms responsible for stimulating mitochondrial proliferation and those responsible for the alterations in mitochondrial morphology have not been clarified to date.

To elucidate the molecular aspects leading to abnormal mitochondrial biogenesis, including mitochondrial proliferation and mitochondrial morphology in the heart of JVS mice, we analyzed the gene expression profile of the heart of JVS and

Abbreviations: JVS, juvenile visceral steatosis; FDD, fluorescent differential display; PDIP38, DNA polymerase delta interacting protein 38; mtSSB, mitochondrial single-stranded DNA-binding protein; mito-DsRed1, mitochondria-targeted DsRed1; GFP, green fluorescent protein; EGFP, enhanced GFP; DMEM, Dulbecco's modified Eagle's medium; PBS, Mg^{2+}- and Ca^{2+}-free phosphate-buffered saline; RT, reverse transcription; PI, propidium iodide; DASPEI, 2-(4-dimethylamino)styryl-N-ethypyrimidium iodide

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control mice by fluorescent differential display (FDD) and Affymetrix GeneChip analyses [9]. In the GeneChip analysis, we found that 176 genes (i.e., 93 known genes and 83 novel genes) were upregulated in JVS mice compared with the levels observed in control mice, and 167 genes (i.e., 67 known genes and 100 novel genes), were downregulated in JVS mice compared with those of control mice. We identified 7 up- and 2 down-regulated genes in JVS mice compared with those of control mice by FDD analysis. We also employed a PCR-select cDNA subtraction method and identified 67 up- and 63 down-regulated genes in JVS mice compared with those of control mice. During the course of investigation of the biological functions of these genes, we found that a mouse homolog of human DNA polymerase delta interacting protein 38 (PDIP38) [10] and mitochondrial single-stranded DNA-binding protein (mtSSB), identified as upregulated genes in the heart of JVS mice, play a role in the mitochondrial fusion and fission reactions, respectively. In addition, we found that these proteins play a role in cell survival.

2. Material and methods

2.1. Mice

Control and JVS mice were maintained as described previously [9,11] and all animal protocols were carried out according to the guidelines for Animal Experimentation at the Institute for Animal Experimentation at the University of Tokushima School of Medicine.

2.2. RNA extraction and fluorescent differential display (FDD)

mRNA was prepared from the hearts of 8-week-old control and JVS mice as described previously [12]. Reverse transcription (RT), PCR, and FDD were performed using mRNA essentially as described [13,14]. RT was performed in a 20 μl volume with 5U of avian myeloblastosis virus reverse transcriptase and 50 pmol of a Rhodamine-labeled]anchor primer; then, cDNA was used for PCR with 10-mer arbitrary oligonucleotide primers (f2, f3, f4, f13, f17, f19, f20 in the TaKaRa FDD kit). The first cycle was performed at 94 °C for 2 min, followed by 40 °C for 5 min, and 72 °C for 5 min, and the following protocol was used for the next 34 cycles: 94 °C for 30 s, 40 °C for 2 min, and 72 °C for 1 min; the final extension was carried out at 72 °C for 5 min. The PCR products were separated on 4% polyacrylamide gel containing 7 M urea and then the results were scanned on a FMBIO II Multi-View fluoroimage analyzer (Takara Bio., Inc.) as described previously [14]. Bands showing differential expression were excised, eluted, and re-amplified with primer sets used for the initial amplification. The PCR products were electrophoresed in agarose gels containing bisbenzimide-PEG conjugate (H.A.-Yellow, Takara Bio. Inc.) in order to separate the PCR-fragments that were identical in length, but differed with respect to base composition [15]. The bands showing differential expression were excised, eluted, and sequenced.

2.3. PCR-select cDNA subtraction method

mRNA was prepared as described above and genes expressed differentially in control and JVS mice were identified by the Clontech PCR-select cDNA subtraction method according to the manufacturer's instructions. The identified clones were sequenced and compared with those in GenBank.

2.4. Plasmid constructs

The coding region of Mitogenin I, embedded in RIKEN FANTOM Clone I, was digested with *Kpn*I and *Psh* BI and subcloned into the pCMS-EGFP (enhanced green fluorescent protein) vector (Clontech, Palo Alto, CA) according to the manufacturer's recommended protocol. For the generation of GFP fusion Mitogenin I constructs, the coding region of the Mitogenin I was amplified with mRNA isolated from the hearts of JVS mice by RT-PCR using gene-specific primers (upper primer, 5′-TTTTCTGGAATGTGAGGTGTGG-3′; lower primer, 5′-CTGGTGGGTAATCCGTGT CTAT-3′) and the products were subcloned into the CT-GFP Fusion TOPO vector, which was designed to enable the fusion of a protein of interest to the Cycle 3 GFP protein (Invitrogen, Carlsbad, CA).

The coding region of mtSSB was amplified with mRNA isolated from the heart of JVS mice by RT-PCR using gene-specific primers (upper primer, 5′-GGAATTCGGCGTGTCCGGAAAAGCCTAA-3′; lower primer, 5′-ATAA-GAATGCGGCCGCTAAACTATTACCCCACAGAGTCCCCCTGAA-3′) and the products were subcloned into pCMS-EGFP or pIRES2-DsRed2 (red fluorescent protein) vectors (Clontech, Palo Alto, CA). For the generation of the GFP fusion mtSSB constructs, the coding region of the mtSSB was amplified with mRNA by RT-PCR using gene-specific primers (upper primer, 5′-CGTG-TCCGGAAAAGCCTAAAGA-3′; lower primer, 5′-CCGCCAACCCTTCCA-ATGAACA-3′) and the products were subcloned into the pcDNA3.1/CT-GFP-TOPO vector, which was designed to enable the fusion of a protein of interest to the Cycle 3 GFP protein.

2.5. Cell cultures and mitochondrial labelling

Mouse C2C12 cells were cultured on 35-mm glass-bottom dishes (IWAKI Scitech, Tokyo, Japan) in 2 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS at 37 °C overnight and the cells were then transfected with pCMS-EGFP-Mitogenin I construct using FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, IN). Two days after transfection, the cells were washed with Mg^{2+}- and Ca^{2+}-free phosphate-buffered saline (PBS), and then the mitochondria were stained with MitoTracker Green (CALBIOCHEM, San Diego, CA). The cells were washed with PBS and the mitochondrial morphology was analyzed under a fluorescent microscope (OLYMPUS IX70, OLYMPUS, Tokyo, Japan) with a color-chilled CCD camera (CoolSNAP Fx10L, Tokyo, Japan). We counted at least 200 cells in each experiments and determined the effects of Mitogenin I on mitochondrial morphology. In addition, the cells were grouped into three categories, class I, class II and class III, and we compared the mitochondria in Mitogenin I-transfected cells with control mitochondria. Class 1 cells showed extensive fragmentation resulting mitochondrial spheres. Class 2 cells showed fragmentation of mitochondria but contained some short mitochondrial rods (<5 μm in length). Cells with any long mitochondrial tubules (>5 μm in length) were placed in Class III. For the flow cytometric analysis, cells that had been cultured in 100-mm dishes were transfected with pCMS-EGFP-Mitogenin I construct. Then, 48 h after transfection, the mitochondria were stained with 2-(4-dimethylamino)styryl-N-ethypyrimidium iodide (DASPEI) (CALBIOCHEM, San Diego, CA) which is taken up in a relatively slow manner as a function of the mitochondrial membrane potential [16]. The cells were then analyzed by a Coulter EPICS XL-MCL flow cytometer (Coulter Corp., Hialeah, FL), as previously described [17].

Effect of mtSSB on mitochondrial morphology was determined as follows. Mouse C2C12 cells were cultured and transfected with mitochondria-targeted DsRed1 (mito-DsRed1) and pCMS-EGFP-mtSSB constructs as described above. Two days after transfection, the cells were washed with PBS, fixed, and were washed with PBS as described above. The mitochondrial morphology was analyzed under a confocal laser-scanning microscope (LSM 5 PASCAL, Carl Zeiss Jena, Jena, Germany) as described above. For the flow cytometric analysis, cells cultured in 100-mm dishes were transfected with pIRES2-DsRed2-mtSSB constructs. Two days after transfection, the mitochondria were stained with MitoTracker Green and then analyzed as described above.

For transfection experiments, we first examined the time course experiments of transfection. We determined the effects of Mitogenin I and mtSSB on mitochondrial morphology at specific time points (24, 48, and 72 h) after transfection, and found that the effects were visible within 24 h after transfection and also observed at 48 and 72 h. In this study, however, transfection experiments were performed at 48 h of transfection to exclude cell damage.

2.6. Subcellular distribution of Mitogenin I and mtSSB

C2C12 cells cultured on the glass-bottom dishes were transfected with mito-DsRed1 and GFP-fusion-Mitogenin I or GFP-fusion-mtSSB constructs. Two