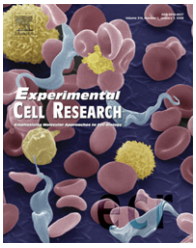
available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/yexcr



Research Article

Human prohibitin 1 maintains the organization and stability of the mitochondrial nucleoids

*Katsumi Kasashima, Megumi Sumitani, Masaaki Satoh, Hitoshi Endo**

Department of Biochemistry, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan

ARTICLE INFORMATION

Article Chronology:
Received 3 August 2007
Revised version received
28 December 2007
Accepted 7 January 2008
Available online 16 January 2008

Keywords:
Mitochondria
Mitochondrial DNA
Prohibitin
Mitochondrial nucleoid
TFAM

ABSTRACT

Mitochondrial prohibitin (PHB) proteins have diverse functions, such as the regulation of apoptosis and the maintenance of mitochondrial morphology. In this study, we clarified a novel mitochondrial function of PHB1 that regulates the organization and maintenance of mitochondrial DNA (mtDNA). In PHB1-knockdown cells, we found that mtDNA is not stained by fluorescent dyes, such as ethidium bromide and PicoGreen, although the mitochondrial membrane potential still maintains. We also demonstrated that mtDNA, which is predominantly found in the NP-40-insoluble fraction when isolated from normal mitochondria, is partially released into the soluble fraction when isolated from PHB1-knockdown cells, indicating that the organization of the mitochondrial nucleoids has been altered. Furthermore, we found that PHB1 regulates copy number of mtDNA by stabilizing TFAM protein, a known protein component of the mitochondrial nucleoids. However, TFAM does not affect the organization of mtDNA as observed in PHB1-knockdown cells. Taken together, these results demonstrate that PHB1 maintains the organization and copy number of the mtDNA through both TFAM-independent and -dependent pathways.

Introduction

Mitochondria are unique organelles that contain genomic DNA not found in the nucleus. In various organisms, mitochondrial DNA (mtDNA) forms nucleoprotein complexes (mitochondrial nucleoids) containing protein components such as mitochondrial transcription factor A (TFAM) and mitochondrial single-stranded DNA-binding protein (mtSSB) (reviewed in [1]). TFAM is a representative component of this complex; mammalian TFAM is known to be involved in packaging [2], maintenance of copy number [3–5], transcription [6,7], and replication of mtDNA [8]. The nucleoid components are believed to regulate stability, replication, transcription, and segregation of mtDNA [1]. Recently, the nucleoid components in *Xenopus* oocytes and HeLa cells were identified [9,10], but the function of each component has not been characterized extensively.

Prohibitin (PHB) proteins (PHB1 and PHB2) are evolutionally conserved mitochondrial proteins localized in the inner membrane, and have various cellular functions including a role in apoptosis [11], cell cycle regulation, transmembrane signal transduction, and control of life span (reviewed in [12]). PHB proteins are also involved in Ras–Raf signaling [13], protection against oxidative stress [14], and innate immunity [15]. Recently, we identified the mitochondrial function of human PHB2, which regulates the stability of mitochondrial proteins such as HS1-associated protein X-1 (Hax-1) and optic atrophy 1 (OPA1), and is involved in anti-apoptosis and regulation of mitochondrial morphology [16]. However, the overall

* *Corresponding author*. Fax: +81 285 44 1827.
E-mail address: hendo@jichi.ac.jp (H. Endo).

0014-4827/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexcr.2008.01.005

functions of PHB proteins in mitochondria have not been clarified completely.

Recent studies indicate that PHB proteins are included in the mitochondrial nucleoid in HeLa cells and *Xenopus* oocytes [9,10], although their involvement in the regulation of mtDNA has not been elucidated. In this study, we identified a novel function of PHB proteins in mitochondria, namely, the involvement of PHB1 in the maintenance of mtDNA. In PHB1-knockdown cells, the status of mtDNA is altered in several ways. First, mtDNA in PHB1-knockdown cells was not stained with either ethidium bromide (EtBr) or PicoGreen, dyes for staining mtDNA in normal living cells, although the mitochondrial membrane potential still maintains. Second, conformation of mtDNA isolated from PHB1-knockdown cells appeared to be altered. Third, mtDNA, which is predominantly isolated in the Nonidet P-40 (NP-40)-insoluble fraction of normal mitochondria, was partially released into the soluble fraction, demonstrating that mtDNA has an altered organization. These phenotypes were not seen in OPA1- or TFAM-knockdown cells, although we revealed that PHB1 regulates the expression of OPA1 [16] and TFAM (in this study) at the protein level. Finally, we found that a slight reduction of mtDNA copy number is observed in PHB1-knockdown cells, which is well consistent with the down-regulation of TFAM protein in these cells. In this paper, we demonstrate for the first time that PHB1 maintains the organization of the mitochondrial nucleoids, independent of the TFAM function, and differentially regulates mtDNA copy number through TFAM stabilization.

Materials and methods

Plasmid construction

For RNA interference (RNAi), small interfering RNA (siRNA) sequences for PHB1 (5′-AACACAGCCTTCCTTCTGCTC-3′), PHB2 (5′-AAGAACCCTGGCTACATCAAA-3′) [16], OPA-1 (5′-GTTATCAGTCTGAGCCAGGTT-3′), Hax-1 (5′-AACCAGAGAGGACAATGATCT-3′), and TFAM (5′-AAGTTGTCCAAAGAAACCTGT-3′) (described in [4]) were introduced into the pSilencer 3.1-H1 Puro vector (Ambion).

Cell culture and transfection

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in an atmosphere containing 5% CO_2. ρ^0 HeLa cells were cultured in RPMI1640 containing 10% fetal bovine serum, pyruvate (0.11 mg/ml), uridine (0.05 mg/ml), and gentamicin (0.05 mg/ml). Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. HeLa cells were plated on 35-mm poly-L-lysine-coated glass-bottomed dishes (Matsunami Glass Ind.). Mitochondria in living HeLa cells were stained with tetramethylrhodamine, ethyl ester, perchlorate (TMRE, 100 ng/ml, Molecular Probes) for 15 min at 37 °C or with rhodamine 123 (0.25 μg/ml) for 30 min at 37 °C. mtDNA was stained with EtBr (2 μg/ml) for 5 min at 37 °C or with PicoGreen solution (3 μl/ml, Molecular Probes) for 60 min at 37 °C. Fluorescent images were captured and analyzed with a μRadiance™ Laser Scanning Confocal Microscope System (BioRad). Cells expressing pSilencer 3.1-H1 Puro constructs were selected with 2 μg/ml of puromycin (Sigma).

Preparation and fractionation of mitochondria

Mitochondria were prepared from HeLa cells as previously described [16]. To assess membrane association, mitochondria suspended in sucrose solution (0.25 M sucrose supplemented with 0.2 mM EDTA pH 7.4) were sonicated on ice. Intact mitochondria were removed by centrifugation at 4 °C for 10 min at 10,000 ×*g*, and the supernatant containing sonicated mitochondria was further centrifuged at 4 °C for 30 min at 100,000 ×*g*. The pellets were collected as the mitochondrial membrane fraction. To assess NP-40-solubility, mitochondria, which contain 200–300 μg of proteins, were suspended in TES buffer (10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 0.25 M sucrose, 0.5% NP-40) on ice for 30 min. After centrifugation at 4 °C for 30 min at 20,000 ×*g*, the membrane pellets and supernatants were separated. The fractions were digested by Proteinase K (100 μg/ml) and RNase A (80 μg/ml) in digestion buffer (10 mM Tris–HCl, pH 8, 25 mM EDTA, 100 mM NaCl, 0.5% SDS) and treated with phenol two times. The mtDNA was purified by ethanol precipitation.

Western blot analysis

Samples were separated by electrophoresis on SDS-polyacrylamide gels (10% or 12% acrylamide) and then electrophoretically transferred to nitrocellulose membranes (Hybond ECL; Amersham Biosciences). The membranes were probed with primary and horseradish peroxidase-conjugated secondary antibodies, and immunoreactive bands were visualized with enhanced chemiluminescence reagents (Amersham Biosciences). The following primary antibodies were used: anti-PHB1 (1:200; NeoMarkers), anti-PHB2 (1:1000; Upstate Biotech.), anti-glyceraldehydes 3-phosphate dehydrogenase (GAPDH, 1:3000; Chemicon), anti-TFAM (1:100; SantaCruz), anti-Porin (1:2000; Calbiochem), and anti-OPA1 (1:1000; [17]).

PCR

For amplification of human 18S ribosomal RNA (rRNA) gene, mitochondrial ND2, and 16S rRNA gene, the following primer sets were used; 18S rRNA: forward, 5′-TTGACGGAAGGGCACCACCAG-3′, reverse, 5′-AGTCAAGTTCGACCGTCTTCTC-3′, ND2: forward, 5′-CACAGCGCTAAGCTCGCACTG-3′, reverse, 5′-GCTAAGATTTTGCGTAGC-3′, 16S rRNA: forward, 5′-TACTACCAGACAACCTTAGC-3′, reverse, 5′-CTTTCTTAATTGGTGGCTGC-3′. Human mtDNA fragment was also amplified using the following primers: forward, 5′-GTCAAAGCGAACTACTATAC-3′ (2872–2891 bp), reverse, 5′-ATTGTTGAAGAGGATAGC-3′. (4687–4704 bp).

Southern blot analysis

Genome DNA from HeLa cells was extracted by standard Proteinase K digestion method described above. Two micrograms of the genomic DNA digested with *Bgl*II and *Pvu*II (mtDNA is linearized by PvuII [18]) was separated on 1% agarose gels, and transferred to Hybond-N^+ (Amersham Biosciences). Labeling of the probe, hybridization, and detection of the signals were done using DIG High Prime DNA labeling and detection system