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Research Article

Maintenance of mitochondrial genome distribution by mitochondrial AAA+ protein ClpX

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ABSTRACT

The segregation of mitochondrial DNA (mtDNA) is important for the maintenance and transmission of the genome between generations. Recently, we clarified that human mitochondrial transcription factor A (TFAM) is required for equal distribution and symmetric segregation of mtDNA in cultured cells; however, the molecular mechanism involved is largely unknown. ClpX is an ATPase associated with various cellular activities (AAA+) proteins that localize to the mitochondrial matrix and is suggested to associate with mtDNA. In this study, we found that RNAi-mediated knockdown of ClpX in HeLa cells resulted in enlarged mtDNA nucleoids, which is very similar to that observed in TFAM-knockdown cells in several properties. The expression of TFAM protein was not significantly reduced in ClpX-knockdown cells. However, the enlarged mtDNA nucleoids caused by ClpX-knockdown were suppressed by overexpression of recombinant TFAM and the phenotype was not observed in knockdown with ClpP, a protease subunit of ClpXP. Endogenous ClpX and TFAM exist in close vicinity, and ClpX enhanced DNA-binding activity of TFAM in vitro. These results suggest that human ClpX, a novel mtDNA regulator, maintains mtDNA nucleoid distribution through TFAM function as a chaperone rather than as a protease and its involvement in mtDNA segregation.

Introduction

Mitochondria contain their own genomic DNA called mitochondrial DNA (mtDNA). Human mtDNA encodes 13 proteins that are essential components of respiratory chain complexes. Mutations and deletions in human mtDNA are closely associated with neuromuscular diseases, such as MELAS [1]. Because mtDNA exists in multiple copies per cell, it is thought that mitochondrial diseases develop if the population of mutant mtDNA molecules is increased and exceeds a critical threshold. mtDNA variants segregate rapidly between generations. Several studies have shown that particular mtDNA variants are not segregated randomly in some human cells [2,3]. The biased segregation of mtDNA variants is suggested to play a role in human mtDNA diseases and mutant load [4,5].

Abbreviations: mtDNA, mitochondrial DNA; AAA+, ATPase associated with various cellular activities; RNAi, RNA interference; TFAM, mitochondrial transcription factor A; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, stroke-like episodes; OPA1, optic atrophy 1; FISH, fluorescent in situ hybridization; mtSSB, mitochondrial single stranded DNA-binding protein; siRNA, small interfering RNA; PHB2, prohibitin 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; EtBr, ethidium bromide; BrdU, bromodeoxyuridine; rRNA, ribosomal RNA; rTFAM, recombinant TFAM

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Recently, Gimap3, a mitochondrial outer membrane GTPase, was identified as the first nuclear encoded gene that regulates tissue-specific mtDNA segregation in leukocytes [6]; however, the molecular basis of the segregation of mtDNA is largely unknown.

mtDNA forms a highly organized structure associated with many proteins. These structures are called mitochondrial nucleoids, and nucleoid components are believed to regulate the stability, replication, transcription, and segregation of mtDNA [7]. Mitochondrial transcription factor A (TFAM) is a major nucleoid protein that binds directly to mtDNA [8]. TFAM regulates transcription of mtDNA and maintains its copy number [8–10]. Recently, we identified a new function of human TFAM; TFAM is required for equal distribution and symmetric segregation of mtDNA in cultured cells [11]. TFAM contains a C-terminal tail abundant in basic amino acid residues, which has been reported to be important for the activation of transcription [12], efficient interaction between TFAM and DNA [13], and proper distribution of mtDNA [11]. How TFAM regulates the distribution and symmetric segregation of mtDNA remains almost unknown.

Mitochondria contain several ATPase associated with various cellular activities (AAA+) proteins [14], and three of them, Lon protease, ClpX, and ATAD3A, are suggested to be included in mitochondrial nucleoids [15]. Actually, Lon is involved in the quality control of oxidative mtDNA [16] and regulates its copy number through selective degradation of TFAM [17]. ATAD3A has been shown to associate with mtDNA and regulates the organization of mitochondrial nucleoids [18]. In this way, mitochondrial AAA+ proteins included in nucleoids play crucial roles in the maintenance of mtDNA. Eukaryotic ClpX is a mitochondrial matrix protein and its physiological function is not largely understood. In prokaryotes, bacterial ClpX has a dual function; it acts as an ATP-dependent chaperone to dissociate protein complexes [19] or as a protease to degrade damaged proteins together with protease subunit ClpP [20]. Because human ClpX can interact with ClpP, as well as bacterial ClpX [21], it is also considered to act either as a chaperone in the absence of ClpP or as an ATP-dependent protease ClpXP with the serine protease ClpP [22]. However, its substrates have not been identified.

In this study, we identified a mitochondrial function of human ClpX: its role in the distribution of mtDNA nucleoids, which appears to be independent of its role in ATP-dependent proteolysis. RNAi-mediated knockdown of ClpX in HeLa cells caused enlarged mtDNA nucleoids, which was confirmed by mtDNA-recognizable fluorescent dye PicoGreen-staining, fluorescent in situ hybridization (FISH), and immunofluorescent analysis against mtSSB and TFAM, core nucleoid factors. Enlarged mtDNA nucleoids are very similar with those we recently observed in TFAM-knockdown cells [11]. However, knockdown of ClpX had a slight effect on TFAM protein levels or mtDNA amounts. Enlarged mtDNA nucleoids caused by ClpX-knockdown were replicatively active and remained present in OPA1-knockdown as well as TFAM-knockdown cells. Supporting their functional relationship, endogenous TFAM and ClpX exist in close vicinity in HeLa cells, and overexpression of recombinant TFAM suppressed the enlarged mtDNA nucleoids phenotype caused by ClpX-knockdown. Furthermore, knockdown of ClpP in HeLa cells hardly induced enlarged mtDNA nucleoids. In in vitro analysis, ClpX was found to enhance the DNA-binding activity of TFAM. These results suggest that ClpX regulates mtDNA nucleoid distribution through TFAM function as a chaperone rather than as a protease, and that TFAM is one of the substrates of ClpX.

Materials and methods

Plasmid construction

For RNA interference (RNAi), small interfering RNA (siRNA) sequences for ClpX (1; 5′-AAGTGAGGGAAGTAGTAAGAA-3′, 2; 5′-AAGTGTGAACTGAATGTTACT-3′), TFAM [23], ClpP (5′-AAGAAG-CAGCTCTATAACATC-3′), OPA1 [23] were introduced into the pSilencer 3.1-H1 Puro vector (Ambion). The coding region of TFAM and its C-terminal deletion mutant (amino acids 1–221) were amplified by PCR from a human heart cDNA library, and the coding region of ClpX was amplified by PCR from cDNA prepared from HeLa cells. These PCR products were introduced into the mammalian expression vector pEF4/Myc-His B (Invitrogen), and the coding sequences containing Myc tag sequences at their 3' termini were also introduced into the mammalian expression vector pIRESpuro3 (Clontech). Mature TFAM (amino acids 42–246) and ClpX (65–633), which lack mitochondrial target sequences, were introduced into the bacterial expression vector pQE-80L (Qiagen).

Cell culture and transfection

HeLa cells were cultured as previously described [23]. Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. HeLa cells were transiently transfected with indicated constructs and were processed four days after transfection in almost all experiments. HeLa cells were plated on 35-mm poly-L-lysine-coated glass-bottomed dishes (Matsunami Glass Ind.). Mitochondria were stained with MitoTracker Red CM-H_2XRos (250 nM, Molecular Probes) for 30 min at 37 °C, and mtDNA nucleoids were stained with PicoGreen solution (3 μl/ml, Molecular Probes) for 60 min at 37 °C. Cells expressing pSilencer 3.1-H1 Puro constructs were selected with 2 μg/ml of puromycin (Sigma). Fluorescent images were captured with the Axio Observer D1 system (Carl Zeiss) or the Leica TCS SP5 confocal microscope system (Leica). For remodeling of nucleoids, HeLa cells were cultured in medium containing 0.5 μg/ml of ethidium bromide (EtBr) for 24 h.

Combined immunofluorescence and fluorescent in situ hybridization

FISH was performed as described previously [11]. The 1.1 kb mtDNA fragment (nt: 16029-599) was labeled with Alexa Fluor 488 fluorescent dye (ARES DNA labeling kit, Molecular Probes) by nick translation (Roche). The probe was used for hybridization at a concentration of about 2 ng/μl in hybridization buffer (50% deionized formamide, 10% Dextran Sulfate, 2 × SSC). HeLa cells were fixed for 15 min with 4% formaldehyde in PBS and then treated for 15 min with 0.1% Triton X-100 in PBS. Cells were then probed with anti-PHB2 rabbit polyclonal antibody (our product) and labeled with Cy3-conjugated anti-rabbit IgG antibody (Molecular Probes). After immunofluorescence, cells were fixed again for 20 min with 4% paraformaldehyde in PBS and then treated for 15 min with 0.1% Triton X-100 in PBS. Cells were treated with RNase A (100 μg/ml in 2 × SSC) for 1.5 h at 37 °C and then dehydrated and rehydrated using 70%, 90%, and 100% ethanol for 2 min at each step. Prehybridization was performed using 2 × SSC buffer (2 × SSC, 0.3 M NaCl, and 0.04 M sodium citrate) for 1 h at 37 °C and cells were dehydrated using a graded series