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Involvement of DNA ligase III and ribonuclease H1 in mitochondrial DNA replication in cultured human cells

Heini Ruhanen [a], Kathy Ushakov [a], Takehiro Yasukawa [a,b,*]

[a] The Wolfson Institute for Biomedical Research, University College London, Gower Street, London, WC1E 6BT, UK
[b] Consortium for Mitochondrial Research (CfMR), University College London, UK

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ABSTRACT

Recent evidence suggests that coupled leading and lagging strand DNA synthesis operates in mammalian mitochondrial DNA (mtDNA) replication, but the factors involved in lagging strand synthesis are largely uncharacterised. We investigated the effect of knockdown of the candidate proteins in cultured human cells under conditions where mtDNA appears to replicate chiefly *via* coupled leading and lagging strand DNA synthesis to restore the copy number of mtDNA to normal levels after transient mtDNA depletion. DNA ligase III knockdown attenuated the recovery of mtDNA copy number and appeared to cause single strand nicks in replicating mtDNA molecules, suggesting the involvement of DNA ligase III in Okazaki fragment ligation in human mitochondria. Knockdown of ribonuclease (RNase) H1 completely prevented the mtDNA copy number restoration, and replication intermediates with increased single strand nicks were readily observed. On the other hand, knockdown of neither flap endonuclease 1 (FEN1) nor DNA2 affected mtDNA replication. These findings imply that RNase H1 is indispensable for the progression of mtDNA synthesis through removing RNA primers from Okazaki fragments. In the nucleus, Okazaki fragments are ligated by DNA ligase I, and the RNase H2 is involved in Okazaki fragment processing. This study thus proposes that the mitochondrial replication system utilises distinct proteins, DNA ligase III and RNase H1, for Okazaki fragment maturation.

1. Introduction

Whilst the majority of cellular DNA is enclosed within the nucleus, mitochondria are also known to contain a separate genome, the mitochondrial DNA (mtDNA). In contrast to diploid nuclear DNA, mtDNA is a multi-copy genome, and in human cells typically 10^3–10^4 copies of mtDNA molecules are present per cell. Human mtDNA is composed of closed circular DNA molecules 16,569 base pairs in length, and encodes 13 subunits of the oxidative phosphorylation complexes and 2 ribosomal RNAs and 22 transfer RNAs for translation of the subunits within mitochondria. All other mitochondrial proteins, including those involved in the replication of mtDNA, are encoded by nuclear genes.

For three decades mammalian mtDNA has generally been considered to replicate *via* a characteristic DNA replication mechanism, a strand-displacement model which entails continuous synthesis of both strands of DNA, without the synthesis of short lagging strand fragments, known as Okazaki fragments [1,2]. In this model, virtually all replication intermediates will be partially single-stranded. However, a series of recent studies has demonstrated that mammalian mtDNA replicates *via* two replication modes, neither of which is identical to the strand-displacement model [3–7]. One has many of the properties of conventional coupled leading and lagging strand synthesis in which both nascent strands are composed of DNA (strand-coupled DNA synthesis). The other mode, designated as RITOLS replication [6], is a novel mechanism: whilst the leading strand is composed wholly of DNA, ribonucleotides are incorporated throughout the lagging strand, and only after considerable delay are they replaced by DNA. The strand-displacement model of mtDNA replication [2,8] was suggested to be based on the partially single-stranded replication intermediates that are generated as a result of ribonucleotide loss from the replication intermediates of RITOLS replication [4,6,7]. Although the reason for the presence of the two replication modes for mtDNA is unclear, a substantial fraction of the mtDNA replication intermediates are products of the strand-coupled DNA synthesis mode, and this mode appears to be predominant when cells amplify mtDNA to restore the copy number after transient mtDNA depletion [3,5]. These findings indicate a significant role for the strand-coupled DNA synthesis mode in the maintenance of mtDNA.

In addition to the duplex theta replication mechanisms described above (the strand-coupled DNA synthesis mode and RITOLS replication mode), which were proposed mainly from work in rodent and chick liver and cultured human cells, the operation of a non-theta replication mechanism was recently implicated in the replication of human cardiac mtDNA [9].

The replication of nuclear DNA employs a coupled leading and lagging strand synthesis mechanism in which the lagging strand is made up with Okazaki fragments. The maturation of Okazaki fragments, in which they are joined together to form a continuous nascent strand,

* Corresponding author. Tel.: +44 20 7679 6613; fax: +44 20 7209 0470.
E-mail address: t.yasukawa@ucl.ac.uk (T. Yasukawa).

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requires two sequential steps. The first step is removal of primer RNA, a short stretch of RNA which functions to prime DNA synthesis, called 'Okazaki fragment processing'. The second step is ligation of the processed 5′ end of the Okazaki fragment and the 3′ end of the next Okazaki fragment. Ribonuclease (RNase) H2, flap endonuclease 1 (FEN1) and the endonuclease/helicase DNA2 are considered to function in the first step [10]. RNase H hydrolyses RNA in the DNA/RNA duplex [11] whilst FEN1 and DNA2 cleave protruding single strands ('flaps') from double stranded DNA molecules. Short flaps are cleaved by FEN1 whereas longer flaps are cleaved by DNA2 [10,12]. Three models have been proposed for Okazaki fragment processing: the RNase H/FEN1 model, the DNA2/FEN1 model and the FEN1-only model (see Reference [10]). These models have been based upon experiments using mammalian and yeast nuclear DNA replication systems, however certain aspects of the molecular mechanisms of each processing pathway appear to vary considerably between the two systems. For instance, whilst the nuclear single-stranded DNA binding protein, replication factor A (RPA), stimulates the endonuclease activity of DNA2 and plays an important role in the sequential cleavage of a flap by the two nucleases in the DNA2/FEN1 pathway in yeast, human DNA2 is inhibited by RPA [12]. On the other hand many similarities also exist, such as during the second step of Okazaki fragment maturation in which ligation of the two adjacent Okazaki fragments is conducted by DNA ligase I in both mammals and yeast [13].

The existence of the strand-coupled DNA synthesis replication mode in mammalian mitochondria suggests that the same proteins required for Okazaki fragment maturation in the nucleus, or proteins functionally related to them, must be necessary in these organelles. RNase H1 [14], FEN1 [15], DNA2 [16,17] and DNA ligase III [18] were reported to be present in mammalian mitochondria. RNase H1 knockout mice exhibited embryonic lethality accompanied by prior depletion of mtDNA, implying the necessity of RNase H1 in mtDNA maintenance during development [14]. However, whether and how RNase H1 is involved in the process of DNA synthesis in mtDNA replication is unknown. FEN1 and DNA2 were recently shown by *in vitro* assays to be capable of processing model substrates that mimic intermediate structures in long-patch base excision repair, and possibly in mtDNA replication [16]. It is unknown, however, whether these proteins function synergistically in Okazaki fragment processing in mammalian mitochondria *in vivo*. Regarding DNA ligase, unlike yeast and plant mitochondria [19,20], mammalian mitochondria do not possess DNA ligase I but do contain vertebrate-specific DNA ligase III [18,21]. DNA ligase III participates in DNA repair in the nucleus [21] and was shown to be required for the maintenance of mtDNA [22,23]. Still, a demonstration of whether DNA ligase III is responsible for the ligation step in mtDNA replication remains elusive.

In this study we established an assay system using cultured human cells to investigate the involvement of the potential Okazaki fragment maturation proteins in mtDNA replication. The expression of target proteins was silenced acutely in living cells using short interferencing RNA (siRNA) during the course of transient mtDNA depletion induced with 2′,3′-dideoxycytidine (ddC) and during the following recovery of mtDNA after ddC removal, where mtDNA replication appears to rely heavily on strand-coupled DNA synthesis. Under these conditions, the recovery of mtDNA copy number and the integrity of mtDNA replication intermediates were investigated using real-time quantitative PCR (rt-qPCR) and neutral two-dimensional agarose gel electrophoresis (2D-AGE). Our data indicate that RNase H1 plays an indispensable role in Okazaki fragment processing in human mtDNA replication, and suggest that DNA ligase III joins Okazaki fragments in human mitochondria.

2. Material and methods

2.1. Cell culture, transfection and ddC treatment

The thymidine kinase 1-deficient human osteosarcoma cell line (143B [TK$^-$]) was cultured in DMEM (Invitrogen, cat. no. 41966) supplemented with FBS, penicillin–streptomycin and 50 μg/ml uridine ('normal medium'). The basic procedure of transfection of cells with short double-stranded RNA (dsRNA) occurred as in Ruhanen et al. [24]. Cells were seeded on the day previous to the initial ddC treatment. On day 1 (Fig. 1A), cells were treated with the normal medium containing 25 μM ddC (Sigma) and incubated for 2 days. On day 3, dsRNA transfection was performed with either control scramble (Sc) dsRNA or a gene-specific dsRNA at a concentration of 3 nM using Lipofectamine 2000 reagent (Invitrogen) in Opti-MEM (Invitrogen). In the case of transfection with two separate dsRNA simultaneously, the concentration of each dsRNA was 1.5 nM. Four hours after transfection, DMEM supplemented with 30% FBS, penicillin–

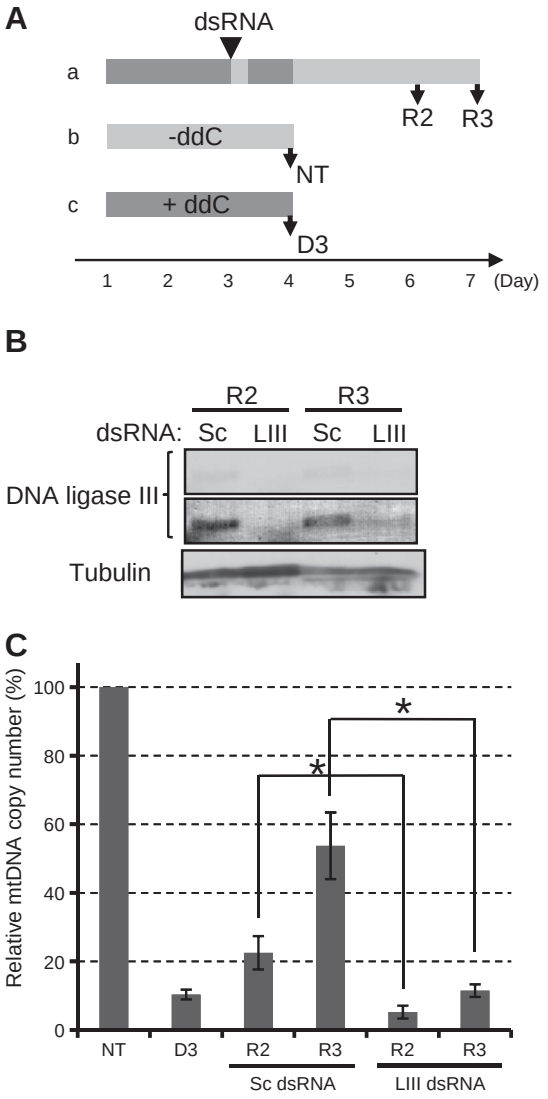

Fig. 1. Knockdown of DNA ligase III delays the recovery of mtDNA copy number. (A) Experimental design. The presence and absence of 2′,3′-dideoxycytidine (ddC) in the medium is indicated by dark and pale grey stripes respectively. (a) Time course of the ddC treatment and dsRNA knockdown. R2 and R3 represent the cell harvest points 2 and 3 days after ddC removal, whilst an arrowhead indicates the timing of dsRNA transfection. (b and c) Incubation of cells in medium in the absence or presence of ddC for 3 days as the non-treated or ddC-treated controls. NT and D3 represent the point of their harvest. (B) Western blot analysis of DNA ligase III levels in cell lysates prepared from cells treated with scramble (Sc) dsRNA or DNA ligase III-specific (LIII) dsRNA. The top panel shows the DNA ligase III band and the middle panel is a digitally enhanced image of the top panel. Tubulin was used as a loading control (bottom panel). (C) The relative copy number of mtDNA analysed with real-time quantitative PCR. The mtDNA content was normalised against nuclear gene content in each sample. The relative mtDNA copy number in NT sample was expressed as 100 in each experiment and those of the other samples were displayed relative to this. Data represent the mean of 3 independent transfection experiments ± SEM. * ($p<0.05$).