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Mitochondrial base excision repair of uracil and AP sites takes place by single-nucleotide insertion and long-patch DNA synthesis

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ABSTRACT

Base excision repair (BER) corrects a variety of small base lesions in DNA. The *UNG* gene encodes both the nuclear (UNG2) and the mitochondrial (UNG1) forms of the human uracil-DNA glycosylase (UDG). We prepared mitochondrial extracts free of nuclear BER proteins from human cell lines. Using these extracts we show that UNG is the only detectable UDG in mitochondria, and mitochondrial BER (mtBER) of uracil and AP sites occur by both single-nucleotide insertion and long-patch repair DNA synthesis. Importantly, extracts of mitochondria carry out repair of modified AP sites which in nuclei occurs through long-patch BER. Such lesions may be rather prevalent in mitochondrial DNA because of its proximity to the electron transport chain, the primary site of production of reactive oxygen species. Furthermore, mitochondrial extracts remove 5′ protruding flaps from DNA which can be formed during long-patch BER, by a "flap endonuclease like" activity, although flap endonuclease (FEN1) is not present in mitochondria. In conclusion, combined short- and long-patch BER activities enable mitochondria to repair a broader range of lesions in mtDNA than previously known.

1. Introduction

Human mitochondrial DNA (mtDNA) is a closed-circular molecule of approximately 16,600 basepairs containing 37 genes which code for 13 polypeptides, 22 tRNAs and 2 rRNAs. All polypeptides are subunits of mitochondrial respiratory complexes of the inner membrane. Although mtDNA only encodes 13 of the ~90 different proteins present in the respiratory chain, it is important for normal cellular function because cells depleted of mtDNA (ρ^0 cells) do not respire normally [1]. Genetically engineered mutator mice that accumulated a substantial number of mutations in mtDNA showed early aging phenotypes and reduced lifespan underlining the significance of mtDNA maintenance [2].

The electron flow during mitochondrial respiration can give rise to reactive oxygen species (ROS) [3]. ROS can cause DNA base lesions and strand breaks, which if left unrepaired may result in mutations and genomic instability [4]. The mutation rate in some regions of human mtDNA, including rRNA and tRNA sequences, is 20–100-fold higher than that of nuclear

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Abbreviations: AP sites, apurinic/apyrimidinic sites; UNG, uracil-DNA glycosylase; BER, base excision repair; SP, short-patch; LP, long-patch; mtDNA, mitochondrial DNA; mtBER, mitochondrial BER; ROS, reactive oxygen species; tRNA, transfer RNA; rRNA, ribosomal RNA; NEM, *N*-ethylmaleimide; FEN1, flap-endonuclease 1; THF, tetrahydrofuran.

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DNA [5]. Somatic and hereditary mutations of mtDNA are associated with a variety of diseases including diabetes and deafness [6,7] cancer [8] and neurodegenerative disorders [9].

DNA polymerase γ (POLγ) is the only DNA polymerase identified in human mitochondria [10]. POLγ is a processive DNA polymerase which consists of two subunits, a large 140 kDa catalytic subunit, POLγA [11] and an accessory factor, POLγB [12]. The large subunit contains a 3′–5′ exonuclease (proof-reading) as well as a dRP lyase activity that removes 5′-deoxyribosephosphate (dRP) moiety during BER [13]. The accessory subunit stimulates the DNA synthesis activity and processivity of POLγ [12,14].

BER is apparently the main mechanism for repair of ROS-generated base lesions in DNA [4]. BER of several oxidative base lesions and uracil have been detected in mitochondria [reviewed in 15]. Nuclear BER in human cells occurs by replacement of a single nucleotide or short-patch repair (SP) or several nucleotides; the so-called long-patch (LP) repair [16]. It is known that the dRP lyase activity of POLβ is unable to cleave modified (oxidized/reduced) moieties [17], the repair of which requires flap endonuclease and LP BER. Given the high rate of ROS production in mitochondria, it is likely that oxidized moieties are continuously formed in mtDNA. How mitochondria deal with DNA damage that requires LP BER in nuclei is not known. BER by enzymes purified from *Xenopus laevis* mitochondria, or by extract from rat liver mitochondria apparently occurs as single-nucleotide insertion [18,19].

Our main aim in conducting this study was to examine the capacity of mitochondria for repair of lesions that in the nucleus require LP BER. First we established an improved method for purification of mitochondria that enabled us to prepare mitochondrial extracts free of detectable nuclear BER proteins. Using these extracts we examined the role of UNG in removal of uracil from mtDNA and carried out mitochondrial BER analysis including, patch-size analysis, and repair of modified AP sites. We found that UNG is the predominant uracil-DNA glycosylase in mitochondria and BER of uracil and AP sites by mitochondrial extracts is in form of SP as well as LP BER.

2. Materials and methods

2.1. Chemicals and antibodies

Synthetic oligonucleotides were purchased from MedProbe (Oslo, Norway). [α-^{33}P]dTTP, [α-^{33}P]dCTP, and [γ-^{33}P]ATP (3000 Ci/mmol) were from Amersham Biosciences. Proteinase K, aphidicolin, *N*-ethylmaleimide (NEM), and Percoll® were from Sigma–Aldrich. Complete® protease inhibitor and T4 DNA ligase were from Roche Inc. Restriction enzymes and T4 polynucleotide kinase were from New England Biolabs. Primary antibodies; APE1 (ab194), APE2 (ab13691), VDAC1 (ab15895), COX IV (ab16056), lamin A + C (ab8984), FEN-1 (ab 462) were all from Abcam Ltd., UK. Antibody to PCNA (PC10, sc-56) was from Santa Cruz Biotechnology, Inc., USA, polyclonal FEN-1 antibody was from Bethyl (BL587), and POLδ (D73020) was from Transduction Laboratories. Neutralizing antibody against the catalytic domain of UNG has been described previously [20]. Paramagnetic protein-A beads were from Dynal, Norway.

2.2. Cell culture

HeLa and HaCaT cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum, 0.03% glutamine, and 0.1 mg/ml gentamicin in 5% CO_2.

2.3. Isolation of crude mitochondria

We harvested the cells by trypsination and washed the cells once with cold PBS and once with an isotonic buffer (20 mM HEPES-KOH pH 7.4, 5 mM $MgCl_2$, 5 mM KCl, 1 mM DTT, and 0.25 M sucrose), resuspended the cells in a hypotonic buffer (20 mM HEPES-KOH pH 7.4, 5 mM $MgCl_2$, 5 mM KCl, and 1 mM DTT) and incubated them on ice for 5–10 min before disruption of the cells by a Dounce homogenizer (5–10 strokes). We immediately added (1:1, v/v) 2× MSH buffer (20 mM HEPES-KOH pH 7.4, 4 mM EDTA, 2 mM EGTA, 5 mM DTT, 0.42 M mannitol, 0.14 M sucrose) to the homogenate to stabilize the mitochondrial membrane as described previously [21]. We centrifuged the homogenate three times at 2000 × *g*, each time for 5 min to separate cell debris and nuclei (the pellet) from mitochondria (the supernatant), and then pelleted the mitochondria at 3000 × *g* for 30 min. The mitochondrial pellet was then resuspended in 1 ml 1× MSH/50% Percoll, the suspension loaded on top of a 1× MSH/50% Percoll gradient (12 ml) and centrifuged at 50,000 × *g* for 1 h at 4 °C. The mitochondria were removed from the gradient and washed once with 1× MSH buffer to remove Percoll, once with 1 ml buffer B (10 mM HEPES-KOH pH 7.4, 0.21 M mannitol, 0.7 M sucrose, and 2.5 mM DTT), resuspended in buffer B containing 1 mg/ml proteinase K in a final volume of 1 ml (unless otherwise is indicated) and incubated at 37 °C for 30 min. The mitochondria were pelleted at 10,000 × *g* for 5 min and washed twice with 0.5 ml of a protease inhibitor mix (0.5 ml protease inhibitor cocktail (1 Complete® tablet dissolved in 1 ml water), 0.5 ml 2× MSH, and 5 mM phenylmethylsulphonyl fluoride (PMSF)). We routinely isolated mitochondria from 30 dishes (150 mm) at 85–90% confluence which after proteinase K treatment yielded on average 0.6–0.8 mg mitochondrial protein.

2.4. Western blot analysis of intact mitochondria

We isolated mitochondria from 30 dishes (150 mm) by Percoll gradient as described above. The crude mitochondrial pellet was resuspended in 0.35 ml buffer B and the suspension divided in seven tubes (0.05 ml each). Proteinase K was added to the samples at the indicated concentrations followed by incubation at 37 °C for 30 min. Proteinase K was inactivated by adding 5 mM PMSF and Complete® protease inhibitor to the samples followed by addition of loading buffer (NuPage) and heating of the samples at 85 °C for 10 min. We separated proteins in 10% denaturing SDS-polyacrylamide gel (NuPage), and transferred them to a PVDF membrane (Immobilon™, Millipore). The membrane was incubated with the primary antibodies at 4 °C overnight, followed by incubation for 1 h at room temperature with either peroxidase-labeled polyclonal rabbit anti-mouse IgG/HRP or peroxidase-labeled polyclonal swine anti-rabbit IgG/HRP (DakoCytomation, Denmark). We incubated the membrane with chemiluminescence reagent (SuperSignali® West Femto Maximum, PIERCE, USA), and visu-