Ung1p-mediated uracil-base excision repair in mitochondria is responsible for the petite formation in thymidylate deficient yeast

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**ABSTRACT**

The budding yeast CDC21 gene, which encodes thymidylate synthase, is crucial in the thymidylate biosynthetic pathway. Early studies revealed that high frequency of petites were formed in heat-sensitive cdc21 mutants grown at the permissive temperature. However, the molecular mechanism involved in such petite formation is largely unknown. Here we used a yeast cdc21-1 mutant to demonstrate that the mutant cells accumulated dUMP in the mitochondrial genome. When UNG1 (encoding uracil-DNA glycosylase) was deleted from cdc21-1, we found that the ung1Δ cdc21-1 double mutant reduced frequency of petite formation to the level found in wild-type cells. We propose that the initiation of Ung1p-mediated base excision repair in the uracil-laden mitochondrial genome in a cdc21-1 mutant is responsible for the mitochondrial petite mutations.

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1. Introduction

An adequate and proper supply of deoxyribonucleoside triphosphates (dNTPs) is crucial for DNA replication and the maintenance of genome integrity [1,2]. In eukaryotic cells, the biosynthesis of dNTPs occurs in the cytoplasm and is normally under tight control such that many enzymes involved in the synthesis of dNTPs are subject to cell cycle regulation [3–6]. Among the four dNTPs, the biosynthetic route of thymidylate is distinctive and has been extensively studied [7]. Thymidylate synthase (TS; EC 2.1.1.45) acts as the key enzyme in the thymidylate biosynthetic pathway and catalyzes the conversion of dUMP to dTMP (Fig. 1). Numerous studies have shown that suppression of TS activity may lead to accumulation of the precursor, dUTP, which enhances misincorporation of dUMP into DNA and arrests DNA replication [8–12].

In the budding yeast, Saccharomyces cerevisiae, the CDC21 gene encodes thymidylate synthase, which is essential for cell viability [13]. Early studies indicated that heat-sensitive cdc21 mutants not only are arrested in nuclear DNA replication but mitochondrial DNA (mtDNA) replication is also stopped immediately after a shift to the restrictive temperature [9,10]. This finding is unexpected because mutations found in cell division control (cdc) mutants impaired in the initiation of the S-phase of cell cycle do not generally have a dramatic impact on mitochondrial DNA, since mtDNA replication is not cell cycle regulated. Furthermore, a very high frequency of respiratory-deficient petites has been observed in this type of mutant, even when grown at the permissive temperature [7,14]. The formation of petites in a cdc21 mutant is likely to be due to the large scale deletion of the mtDNA [14]. Loss of mtDNA integrity in a cdc21 mutant at the permissive temperature suggests that the stability of mtDNA is more sensitive to thymidylate starvation than nuclear DNA.

The yeast cell lacks thymidine kinase and is not normally permeable to exogenous thymidine or its derivatives [15,16]; thus the TS catalyzed reaction is the sole source of thymidylate for both mtDNA and nuclear DNA synthesis. A single amino acid change at the residue 139 of the Cdc21p from glycine to serine creates a temperature-sensitive allele of cdc21-1 that is defective in the TS activity [17]. In cdc21-1, the frequency of petite formation reaches almost 100% after only a few cell generations [14]. In this study, we used the yeast cdc21-1 mutant to demonstrate that mutant cells accumulated dUMP in the mitochondrial genome as a result of the impediment in thymidylate synthesis. The incorporated uracil in the genome is normally subject to Ung1p-mediated base excision repair (BER) to rectify the abnormal incorporation [11,18,19]. Unexpectedly, we have found that the initiation of
BER to repair the uracil-laden mitochondrial genome was responsible for petite formation in cdc21-1.

2. Materials and methods

2.1. Yeast media, growth conditions, strains and genetic manipulations

Yeast cultures were routinely grown in YEPD (1% yeast extract, 2% peptone, 2% dextrose) and YEPG (1% yeast extract, 2% peptone, 3% glycerol) as needed [20]. The yeast strain BY20612 (Matα cdc21-1 ade1 cdc2 ura3 his7 lys2 tyr1 gal1) with a genetic background of A364A was obtained from YGRC, Osaka, Japan. JY781 (Matα ura3 leu2 his3 lys2) and JY782, dissected for haploid spores of A364A was obtained from YGRC, Osaka, Japan. JY781, JY782, and BY20612 were verified and shown to be identical except for petite formation. Only a small (2) subunits; DUT1: dUTPase; CDC21: thymidylate synthase; CDC8: thymidylate kinase. NDPK indicates a universal enzyme nucleoside diphosphate kinase.

Fig. 1. The biochemical pathway of dTTP biosynthesis in S. cerevisiae. Only pertinent intermediates in the last few steps of the pathway are shown. DNA polymerases are able to use dTTP or dUTP during replication and incorporate them into DNA. Under normal conditions, dTTP is incorporated in DNA much more frequently than dUTP (black solid arrow versus dashed arrow, respectively). Genes encoding the various enzymes involved in this pathway are as follows: URA6: uridylate kinase; RNR1,2: ribonucleotide reductase large (1) and small (2) subunits; DUT1: dUTPase; CDC21: thymidylate synthase; CDC8: thymidylate kinase. NDPK indicates a universal enzyme nucleoside diphosphate kinase.

2.2. Plasmid construction

To create MLSA-UNG1, two primer pairs were designed, according to a previous report [22], to PCR-amplify the truncated UNG1 DNA fragment without the first 16-residue coding sequence. MLSA and the whole-length UNG1 clone was also obtained in a similar way by PCR amplification.

2.3. Cell cycle analysis by flow cytometry

The protocol of yeast cell cycle analysis has been described previously [20]. Briefly, logarithmic phase of yeast cells were harvested, fixed in 70% ethanol, washed and resuspended in 50 mM sodium citrate, pH 7.5. About 1 × 10⁶ cells were adjusted to a volume of 0.5 ml with the same buffer. Each sample had RNase A added and then was incubated at 50 °C for 1 h, which was followed by the addition of Proteinase K to remove proteins. After further incubation for 1 h at 50 °C, propidium iodide was added. Samples were finally analyzed using a FACS Calibur (BD Biosciences) system and the results were analyzed using Cell Quest software.

2.4. Measurement of the petite formation frequency

The measurement of petite formation followed established procedures [24]. Single colonies were inoculated in YEPD medium and grown to stationary phase. Appropriate dilutions of the cells were plated in triplicate on YEPD plates, replica plated onto YEPG plates after colonies were formed and then incubated for an additional 4 days at 23 °C before observation. The percent of respiration-incompetent colonies, defined as petites, was calculated as the percent of total colonies (on YEPD) that failed to grow on YEPG.

2.5. Measurement of the uracil in the yeast genomic DNA

Total genomic DNA from yeast nuclei and mitochondria were extracted and separated according to described methods with minor modifications [25]. Briefly, yeast cells were digested to form spheroplasts by adding lyticase. Spheroplasts were lysed in solution containing sarcosyl and clarified at 15 000 × g. The supernatant was collected and both nuclear DNA and mtDNA were banded on a CsCl-bis-benzimide gradient.

For the uracil measurement [26], aliquots of DNA were set up in a final volume of 22.5 μl with the addition of Escherichia coli uracil–DNA glycosylase (NEB, USA) and incubated for 30 min at 37 °C. The reaction was stopped and denatured by adding 10× alkaline electrophoresis buffer and 6× alkaline loading buffer. Samples were loaded onto a 0.8% alkaline agarose gel for electrophoresis at 2.8 V/cm for 4.5 h. The gel was then neutralized and stained with ethidium bromide for imaging.

3. Results and discussion

3.1. S-phase delay and petite formation contribute to slow growth in cdc21-1

One of the major phenotypes associated with the cdc21-1 mutant is the strain’s poor growth rate. The estimated generation time of cdc21-1 at the permissive temperature was over 4 h in rich medium compared to 2 h for wild-type cells (Table 1). The poor growth rate of cdc21-1 manifested as a delayed S-phase during the cell cycle. As shown in Fig. 2A, an exponentially growing culture of cdc21-1 accumulated around 40% of cells in the S-phase compared to 20% of wild-type cells. The cell cycle delay resulting from nucleotide deprivation has been notably exemplified by the use of hydroxyurea to block DNA synthesis in a wide variety of cells, including budding yeast [27]. Hydroxyurea is used to inhibit the enzyme ribonucleotide reductase and, as a result, deprives the cells of their ability to synthesize deoxyribonucleotides [27,28]. In yeast, nucleotide depletion triggers replication fork stalling and cell cycle arrest through induction of the S-phase checkpoint proteins, namely the Meclp and Rad53p protein kinases [29–31]. Checkpoint mutants are, therefore, sensitive to hydroxyurea [29,30]. Consistent with this notion, we found that cdc21-1 was lethal in combination with either the mecl-1 or the rad53-11 mutative allele when growing at the permissive temperature (unpublished results). The other important factor contributing to the slow growth of cdc21-1 was the high frequency of petite formation in the mutant culture [14]. Respiratory-deficient (petite) mutants of yeast are characterized by the formation of small colonies due to a slower generation time (min) 110 ± 18 269 ± 20 115 ± 12 184 ± 10

<table>
<thead>
<tr>
<th>Strains</th>
<th>WT</th>
<th>cdc21-1</th>
<th>ung1A</th>
<th>ung1A cdc21-1</th>
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<tr>
<td>Generation time (min)</td>
<td>110 ± 18</td>
<td>269 ± 20</td>
<td>115 ± 12</td>
<td>184 ± 10</td>
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*a* The yeast strains were WT (JY781), cdc21-1 (JY789), ung1A (JY950) and ung1A cdc21-1 (JY955). The growth rate was measured at 23 °C. The generation time was calculated from three independent experiments for each strain.
cellular growth and by an inability to utilize non-fermentable carbon sources such as glycerol for growth [32]. We grew yeast cultures in YEPG to maintain mitochondrial competence as needed and to measure the frequency of petite formation. As shown in Fig. 2B, cdc21-1 failed to grow on YEPG at all and displayed 100% petite formation. In contrast, the average petite frequency of the wild-type strains in our experiments was around 5%. Overall, these results indicated that cdc21-1 suffered a slow growth rate from both S-phase checkpoint-mediated cell cycle delay and because it produces petite mutations at a high rate.

3.2. Incorporation of uracil into the cdc21-1 mitochondrial genomic DNA

Increasing levels of dUTP and its incorporation into DNA have been proposed as a major genetic consequence of thymidylate starvation in the nucleus [8,26]. It has been suggested that cdc21-1 has rho− phenotype, whose petite mutants contain mtDNA and are made up of mainly deletion mutations [14]. Therefore, we determined whether the incorporation of uracil also occurred in the mitochondrial genome of cdc21-1 and whether this might lead to mtDNA aberrations. To this end, we measured the uracil content in yeast DNA isolated from both wild-type and cdc21-1 cells.

Since the endogenous yeast uracil–DNA glycosylase, Ung1p, might be able to efficiently remove all incorporated uracils during the extraction procedure, we purified DNA from both wild-type and cdc21-1 yeast strains with an ung1Δ background [11,12,26]. We then added E. coli uracil–DNA glycosylase, Ung, to the purified DNA to remove all the uracils and to leave abasic sites in the DNA. Subsequently, alkaline agarose gel electrophoresis was used to break the nicked DNA so that it was possible to observe the frequency of breaks, which would correspond to the amount of uracils incorporated into the DNA. As shown in Fig. 3A and B, both the nuclear and mitochondrial genomic DNA from the ung1Δ cdc21-1 yeast cells were broken down into smaller fragments with a faster gel mobility after Ung treatment. This contrasted with the DNA from the wild-type cells (ung1Δ), which was unchanged after the treatment. The average size of the digested DNA fragments was ~500 nucleotides in the nuclear DNA of the ung1Δ cdc21-1 double mutant (Fig. 3A). This result indicates that the steady-state level of uracils in this mutant is ~5 × 10^4 uracils per haploid genome of S. cerevisiae. The estimated level of substitution of uracil for thymine, therefore, equals to ~1%. In addition, the distribution of mtDNA fragments in the same double mutant showed much broader range from 2 to 5 kb in size (Fig. 3B). This may reflect the variations of mitochondrial genome in yeast. Given the average yeast mitochondrial genome size of ~85 kb, this would also translate into 30–80 uracils per mitochondrial genome or 0.05–0.1% of uracil substitution for thymine in mtDNA. These results strongly support the hypothesis that, in contrast to wild-type cells, uracil is
addition, we applied the canavanine resistance assay to measure the spontaneous mutation rate in both cdc21-1 and cdc21-1 ung1Δ strains and did not observe difference between these two strains (data not shown). Therefore, the deletion of the UNG1 gene in cdc21-1 did not result in any further growth defect as compared to the single mutant. Nor did it have any impact on the nuclear behavior of cdc21-1 with respect to cell cycle progression.

Petite formation has been found earlier to be one of the factors contributing to the slow growth of cdc21-1 and therefore we then examined petite formation by the double mutant when grown in YEPG medium. The result showed that the double mutant not only grew well in YEPG but the petite formation frequency was dramatically reduced to a level similar to that of the wild-type cells (Fig. 2B). Since Ung1p plays a key role in uracil-BER, our results strongly suggest that Ung1p mediated uracil-BER may be responsible for petite formation in cdc21-1.

Incorporation of dUMP during replication results in U/A pairs, which are generally not miscoding or mutagenic per se, but may give rise to abasic (AP) sites, which are generated during uracil-BER [26,33]. Without proper processing, these AP sites then can lead to single-strand DNA breaks or gaps that act as substrates for various genetic mutations [18,34,35]. Unlike the mtDNA defects that are associated with the cdc21-1 strain, the nuclear mutation rate of the cdc21-1 strain does not seem to be higher than that of wild-type cells [14]. Thus, it is likely that the uracil-BER activity present in the nucleus is more efficient than that found in the mitochondria.

Previous studies have already shown that the deletion of UNG1 is able to protect yeast cells from the deadly consequences of cytotoxic drugs that deplete thymidylate or the effects of erroneous thymidylate metabolism [11,18,26]. In this report, we have provided the first evidence to show that the ung1 null mutation is also able to rescue the yeast cells from the mitochondrial dysfunctions caused by thymidylate deficiency. Interestingly, the effect of UNG1 deletion by which it prevents mitochondrial dysfunction in cdc21-1 seems to be UNG1-specific. Deletion of two other known mitochondrial DNA glycosylase genes, NTG1 and OGG1, which are responsible for the BER of oxidatively damaged mtDNA in yeast [36], did not reduce petite formation in cdc21-1 at all (data not shown).

3.4 Mitochondrial, not nuclear, Ung1p-mediated BER triggers mtDNA mutations

Unlike human cells, which have distinct nuclear and mitochondrial forms of the Ung proteins, the yeast cell contains only one Ung1p [37]. Yeast Ung1p is predominantly a nuclear protein, although it has been found that Ung1p can be distributed into the cytoplasmic mitochondria as well [22,38]. The localization of Ung1p into mitochondria depends on the presence of a 16-residue mitochondrial localization signal (MLS) in the N-terminus of the protein. Deletion of MLS results in Ung1p localizing exclusively in the nucleus [22]. To further demonstrate that only mitochondrial Ung1p is responsible for petite formation in cdc21-1, we constructed full-length UNG1 and a MLSA-UNG1 form with the 16-residues of the MLS deleted using a yeast integrating-type vector, pRS303. Both the pRS303-MLS-A-UNG1 and pRS303-UNG1 plasmid DNAs were then integrated into the ung1Δ cdc21-1 double mutant. As shown in Fig. 4, the double mutant transformed with an empty vector showed good growth in YEPG and had a low petite formation frequency as expected. When the full-length UNG1 was expressed in the double mutant, the transformed cells lost mitochondrial function again and became similar to the cdc21-1 mutant alone; specifically, they were unable to grow on YEPG and petites occurred at high frequency (Fig. 4A and B). On the other hand, when MLSA-UNG1 was introduced, the cells retained their normal mitochondrial function and grew rather well on YEPG (Fig. 4A and B).

### Figure 3

The cdc21-1 mutant accumulates uracil in the nuclear and mitochondrial genomic DNA. Nuclear genomic DNA (A) and mitochondrial genomic DNA (B) of the exponential growing WT (in ung1Δ background, JY950) and cdc21-1 (in ung1Δ background, JY955) cells were extracted, treated with (+) or without (−) the E. coli Ung enzyme and then subjected to alkaline gel electrophoresis analysis as described in Section 2. Molecular weight markers in kilobases (kb) are indicated.

### Figure 4

A UNG1 deletion prevents mtDNA mutations in cdc21-1

During the process of preparing the ung1Δ cdc21-1 double mutant, we noticed that the spores of ung1Δ cdc21-1 consistently produced a larger colony size than the spores of cdc21-1. This result prompted us to examine the growth rate of the double mutant. The generation time of the double mutant in rich medium was around 3 h at 23°C, almost 1 h faster than cdc21-1 (Table 1). However, they were still temperature sensitive, namely they are unable to grow at 36°C and also possessed exactly the same cell cycle profile as cdc21-1 with a characteristically delayed S-phase (Fig. 2A). In
These results unambiguously demonstrate that only the mitochondrial Ung1p mediated uracil-BER is responsible for the profound genetic aberrations that occur in mtDNA during thymidylate deficiency.

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