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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.

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 mutant 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

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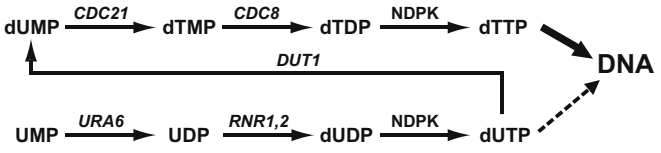

Fig. 1. The biochemical pathway of dTTP biosynthesis in *S. cerevisiae*. Only pertinent intermediates in the last few steps of the pathway are shown. DNA polymerases are able to use dTTP or dUTP during replication and incorporate them into DNA. Under normal conditions, dTTP is incorporated in DNA much 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.

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 (*MAT*a *cdc21-1 ade1 ade2 ura1 his7 lys2 tyr1 gal1*) with a genetic background of A364A was obtained from YGRC, Osaka, Japan. JY781 (*MAT*a *ura3 leu2 his3 lys2*) and JY782 (*MAT*α *ura3 leu2 his3 trp1*) were two wild-type strains used in the laboratory. A *cdc21-1* DNA fragment was PCR-amplified from the genomic DNA of BY20612 and transformed into JY781 to obtain an isogenic *cdc21-1* mutant strain, JY789, by the gene replacement method [21]. The cell growth characteristics of JY789 and BY20612 were verified and shown to be identical except auxotrophic markers. JY789 was crossed with JY782 to form diploid, JY789 × JY782. One copy of the *UNG1* genes was then deleted directly from the diploid, JY789 × JY782, dissected for haploid spores in order to generate the strains JY950 (*MAT*a *ura3 leu2 his3 ung1Δ::LEU2*) and JY955 (*MAT*a *ura3 leu2 his3 cdc21-1 ung1Δ::LEU2*). These processes followed established procedures [21].

2.2. Plasmid construction

To create MLSΔ-*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, MLS, and this was subcloned into a yeast vector, pRS303 [23]. The full-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 1×10^7 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 sarkosyl 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, it 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 Mec1p 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 *mec1-1* 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

Table 1
The growth rate of various yeast strains.[a]

Strains	WT	*cdc21-1*	*ung1Δ*	*ung1Δ cdc21-1*
Generation time (min)	110 ± 18	269 ± 20	115 ± 12	184 ± 10

[a] The yeast strains were WT (JY781), *cdc21-1* (JY789), *ung1Δ* (JY950) and *ung1Δ cdc21-1* (JY955). The growth rate was measured at 23 °C. The generation time was calculated from three independent experiments for each strain.