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Checkpoint deficient *rad53-11* yeast cannot accumulate dNTPs in response to DNA damage

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Abstract

Deoxyribonucleotide pools are maintained at levels that support efficient and yet accurate DNA replication and repair. Rad53 is part of a protein kinase regulatory cascade that, conceptually, promotes dNTP accumulation in four ways: (1) it activates the transcription of ribonucleotide reductase subunits by inhibiting the Crt1 repressor; (2) it plays a role in relocalization of ribonucleotide reductase subunits RNR2 and RNR4 from nucleus to cytoplasm; (3) it antagonizes the action of Sml1, a protein that binds and inhibits ribonucleotide reductase; and (4) it blocks cell-cycle progression in response to DNA damage, thus preventing dNTP consumption through replication forks. Although several lines of evidence support the above modes of Rad53 action, an effect of a *rad53* mutation on dNTP levels has not been directly demonstrated. In fact, in a previous study, a *rad53-11* mutation did not result in lower dNTP levels in asynchronous cells or in synchronized cells that entered the S-phase in the presence of the RNR inhibitor hydroxyurea. These anomalies prompted us to investigate whether the *rad53-11* mutation affected dNTP levels in cells exposed to a DNA-damaging dose of ethylmethyl sulfonate (EMS). Although dNTP levels increased by 2- to 3-fold in EMS treated wild-type cells, *rad53-11* cells showed no such change. Thus, the results indicate that Rad53 checkpoint function is not required for dNTP pool maintenance in normally growing cells, but is required for dNTP pool expansion in cells exposed to DNA-damaging agents.

Keywords: Rad53; dNTP; DNA damage; RNR; EMS; Yeast

Cells have evolved mechanisms to maintain or augment deoxyribonucleotide triphosphate levels during DNA synthesis and repair. Yeast cells respond to DNA damage and replication block by arresting the progression of the cell cycle at specific points and by inducing the expression of genes thought to facilitate DNA repair. These responses are mediated by a kinase cascade that appears to be conserved among eukaryotes. Two essential genes, *MEC1* and *RAD53*, are central players in the kinase cascade that leads to cell-cycle arrest at all the checkpoints and transcriptional activation in response to DNA damage. Cells with mutations in the Mec1 or Rad53 genes are defective in both cell-cycle arrest and gene expression responses in response to DNA damage, but retain their essential functions for cell survival under normal conditions [1–5]. Among the best characterized transcriptional targets of the Rad53 kinase cascade are the RNR genes, which encode subunits of ribonucleotide reductase, the enzyme that catalyzes the rate-limiting step in dNTP synthesis [1]. In addition to transcriptional induction of RNR genes, the Rad53 pathway also induces RNR activity through the removal of Sml1, an RNR inhibitor that binds to large subunits and inhibits enzyme activity [6]. Lethality of Rad53 null mutation is suppressed by overexpression of genes encoding ribonucleotide reductase [3] or by deletion of RNR inhibitor Sml1 [6]. Thus, it could be concluded that essential function of the Rad53 pathway is to maintain dNTP pools.

Although many studies have investigated the mechanisms of RNR regulation, relatively few have determined

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the consequence of such regulation, namely, the effect on dNTP levels. One exception is a study showing that *sml1* mutants have levels of dNTPs that are 2.5-fold higher than wild-type cells [6]. Given that Rad53 inhibits Sml1, we were surprised that a checkpoint-deficient *rad53-11* allele did not result in lower dNTP levels in asynchronously growing cells [7]. Loss of function mutations in Rad53 would be expected to stimulate Sml1, inhibit RNR activity and lower dNTP levels. Although mutation of Sml1 has been shown to increase dNTP levels, it has never been established that mutation of Rad53 has a reciprocal effect on dNTP levels. Furthermore, Rad53 stimulates transcription of the genes required for dNTP synthesis, including RNR1, 2, and 4 [1]. Thus, mutation of Rad53 would also be expected to have a transcriptionally mediated inhibitory effect on dNTP levels. To test these expectations, we determined the levels of dNTP in wild-type and *rad53-11* yeast that were treated with EMS to induce DNA damage. While EMS treatment led to a 2- to 3-fold increase in the size of all four dNTP pools within wild-type cells, *rad53-11* mutants did not exhibit increased dNTP pools which suggests that the Rad53 pathway is required for dNTP accumulation upon exposure to DNA damage.

Materials and methods

Yeast cells, growth and EMS treatment. Cells were grown in YPD media. Wild-type strain MY377 (MAT-a ade2 ura3 leu2 trp1 his3 bar1) and rad53-11 mutant strain MY376 (MAT-a ADE2 ura3 leu2 trp1 his3 rad53-11 pep4:URA3 bar1) have been previously described [7].

Ethylmethylsulfonate (EMS) mutagenesis was done as described by Machado et al. [8].

dNTP pool measurements. Approximately 3×10^8 cells were harvested and extracted as described by Muller [9]. Each precipitated sample was resuspended in 200 μl of cold H_2O and assayed for each of the four dNTPs by the DNA polymerase-based enzymatic assay [10], which is based on the incorporation of a limiting dNTP into an alternating co-polymer template poly(dA–dT) or poly(dI–dC) by Klenow DNA polymerase in the presence of an excess of ^{3}H-labeled complementary dNTP.

Results

To assess the role of the Rad53 pathway in dNTP accumulation, we first tested whether EMS treatment would elevate the size of the dNTP pools in wild-type cells. Asynchronously growing wild-type cells (MY377) were treated with 0.01% EMS, a DNA alkylating agent, and levels of all four dNTPs were measured. As shown in Fig. 1A, the dNTP pools in untreated cells were 256 ± 8 pm/10^8 cells for dTTP, 476 ± 44 pm/10^8 cells for dATP, 153 ± 8 pm/10^8 cells for dCTP and 130 ± 8 pm/10^8 cells for dGTP. After 1-h EMS treatment, the dNTP pools were 746 ± 20 pm/10^8 cells for dTTP, 476 ± 44 pm/10^8 cells for dATP, 351 ± 4 pm/10^8 cells for dCTP and 256 ± 21 pm/10^8 cells for dGTP. Thus, EMS-induced DNA damage led to a 2- to 3-fold increase in dNTP pool sizes in wild-type cells.

We next considered whether DNA damage would have an effect on the size of the dNTP pools in *rad53-11* mutants. As shown in Fig. 1B, the dNTP pools in untreated asynchronously growing *rad53-11* mutants were 420 ± 8 pm/10^8 cells for dTTP, 167 ± 10 pm/10^8 cells for dATP, 151 ± 1 pm/10^8 cells for dCTP and 123 ± 3 pm/10^8 cells for dGTP. After 1-h EMS treatment, the dNTP pools were 426 ± 9 pm/10^8 cells for dTTP, 182 ± 7 pm/10^8 cells for dATP, 129 ± 3 pm/10^8 cells for dCTP, and 126 ± 2 pm/10^8 cells for dGTP. Unlike wild-type cells, *rad53-11* mutants were not able to elevate their dNTP pools in response to EMS-induced DNA damage, which suggested that an intact Rad53 pathway was required for dNTP accumulation after DNA damage.

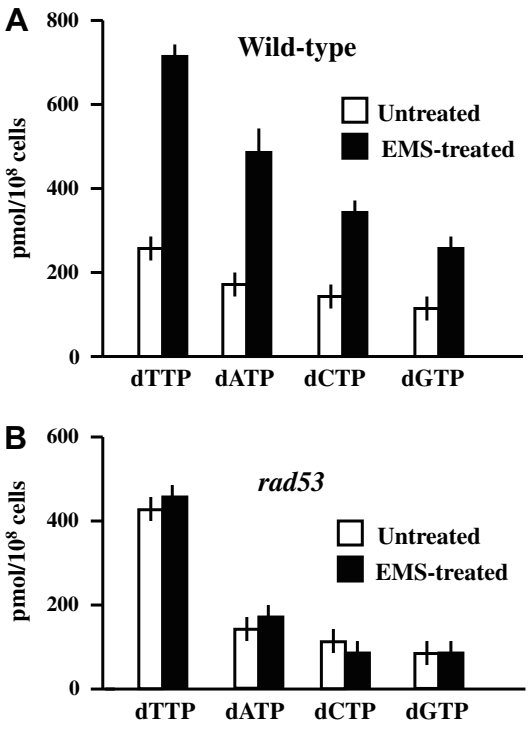

Fig. 1. Deoxyribonucleotide triphosphates levels in wild-type (WT) and *rad53-11* yeast before and after EMS-induced DNA damage. WT (MY377) and *rad53-11* mutants cells (MY376) were grown in YPD at 30 °C to a density of 10^7 cells/ml. Cultures were split, incubated with or without EMS (0.01%) for 1 h at 30 °C, and then harvested for dNTP pool measurements. (A) dNTP pools of WT cells in the absence or presence of EMS. (B) dNTP pools of *rad53-11* cells in the absence or presence of EMS. Error bars represent the range of duplicate determinations.

Discussion

In yeast, the convergence of many different RNR regulatory mechanisms to the Rad53 pathway highlights the role of the pathway in dNTP synthesis in response to DNA damage. However, there has been no study showing a direct relationship between the level of dNTPs and the Rad53 pathway. Our study revealed that the Rad53 pathway is necessary for dNTP accumulation in response to DNA damage.

It has been shown that cell survival after DNA damage is directly linked to elevated levels of dNTPs in yeast [11].

Mammalian cell response to DNA damage is somewhat ambiguous since neither logarithmically growing nor synchronized cells show accumulation of dNTP pools after DNA damage [12]. Our data are consistent with the idea that cells accumulate dNTPs in response to DNA damage as suggested by Chabes et al. [11]. Although the DNA damage response and dNTP synthesis pathways of yeast and mammals have significant similarities, it is neither clear why mammalian cells do not show an increase in the sizes of their dNTP pools nor how they perform the repair process in the event of DNA damage.

Our dNTP pool measurements in wild-type and *rad53-11* mutant cells were consistent with previously published results [7,13–15]. In one of our previous studies, similar dNTP levels were observed for asynchronous *rad53-11* cells, and mutants did not show any cell-cycle perturbations when analysed by flow cytometry, which suggested that *rad53-11* cells operate under a normal cell-cycle progression. However, the magnitude of the increase in dNTP levels in synchronized wild-type yeast as cells progressed through the S-phase was more significant than that of synchronized *rad53-11* cells. This shows that the Rad53 pathway plays a role in induction of dNTP synthesis during a normal S-phase [7]. In spite of having slightly lower amount of dNTPs, the normal cell-cycle progression of *rad53-11* cells also shows that the level of dNTPs is above the threshold level required for efficient DNA synthesis during a normal S-phase.

Normal transcriptional regulation of RNR is governed by a cell-cycle-dependent mechanism exaggerated by *cis*-acting-elements (MCB elements) which ensure expression at the late G1-/S-phase of the cell cycle [1,16]. The *RAD53* gene also contains two similar *cis*-acting-elements in its promoter that up-regulate its expression at late G1-/S-phase [17]. Cell-cycle regulation of Rad53 may be important for S-phase specific inhibition of Sml1 to release RNR activity during a normal S-phase. But, in response to DNA damage it probably shows its effect through a combined mechanism involving RNR transcriptional activation, Rnr2 and Rnr4 re-localization and Sml1 inhibition which together may ensure that the cells synthesize an adequate amount of dNTPs to repair DNA damage (Fig. 2). We have previously shown that when DNA replication is blocked by a *dbf4* mutation, cells hyper-accumulate dNTPs upon entry into the S-phase [14]. Based on this observation, a possible explanation of how wild-type cells accumulated dNTPs in response to EMS treatment, while *rad53-11* cells did not, is that exposure to EMS triggers an S-phase specific checkpoint which halts replication fork progression and stops dNTP consumption during the S-phase in wild-type cells but not in *rad53-11* cells. Thus, further studies should be performed to understand these Rad53-dependent mechanisms and to determine to what extent they play a role for increasing dNTP levels in response to DNA damage.

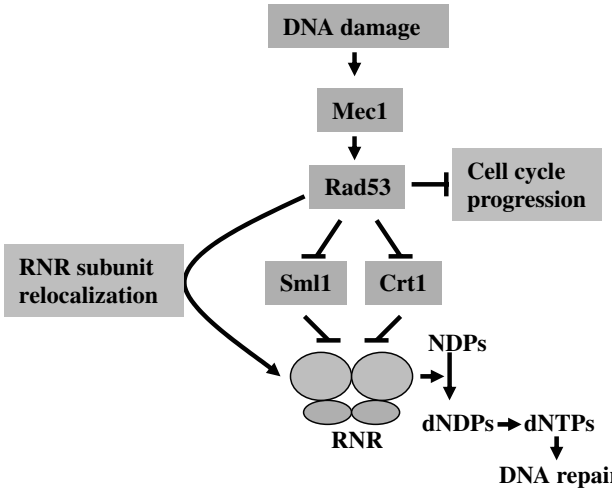

Fig. 2. A model showing the role of the Rad53 pathway in dNTP accumulation in response to DNA damage (intermediate players are not shown). It is hypothesized that cessation of DNA replication, upregulation of RNR via transcriptional induction, Sml1 inhibition and RNR subunit relocalization lead to dNTP accumulation.

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