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Sensor and effector kinases in DNA damage checkpoint regulate capacity for homologous recombination repair of fission yeast in G2 phase

Shinji Yasuhira[a,*], Takeshi Saito[c], Chihaya Maesawa[a], Tomoyuki Masuda[b]

[a] Department of Tumor Biology, Institute of Biomedical Sciences, Iwate Medical University, Iwate 028-3694, Japan
[b] Department of Pathology, School of Medicine, Iwate Medical University, Iwate 028-3694, Japan
[c] Division of Radiation Life Science, Research Reactor Institute, Kyoto University, Osaka 590-0494, Japan

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ABSTRACT

Although the G2/M DNA damage checkpoint is currently viewed as a set of coordinated cellular responses affecting both cell cycle progression and non-cell cycle targets, the relative contributions of the two target categories to DNA repair and cell survival after exposure to ionizing radiation have not been clearly addressed. We investigated how *rad3* (*ATR* ortholog) or *chk1*/*cds1* (*CHK1*/*CHK2* orthologs) null mutations change the kinetics of double-strand break (DSB) repair in *Schizosaccharomyces pombe* cells under conditions of forced G2 arrest. After 200-Gy γ-ray irradiation, DSBs were repaired in *rad3Δ cdc25-22* or *chk1Δ cds1Δ cdc25-22* cells, almost as efficiently as in *cdc25-22* cells at the restrictive temperature. In contrast, little repair was observed in the checkpoint-deficient cells up to 4 h after higher-dose (500 Gy) irradiation, whereas repair was still efficient in the control *cdc25-22* cells. Immediate loss of viability appeared not be responsible for the repair defect after the higher dose, since both checkpoint-proficient and deficient cells with *cdc25-22* allele synchronously resumed cycling with a similar time course when released to the permissive temperature 4 h after irradiation. Recruitment of repair proteins Rad11 (Rpa1 ortholog), Rad22 (Rad52 ortholog), and Rhp54 (Rad54 ortholog) to the damage sites was not significantly impaired in the checkpoint-deficient cells, whereas their release was profoundly delayed. Our results suggest that sensor and effector kinases in the damage checkpoint machinery affect the efficiency of repair downstream of, or in parallel with the core repair reaction.

1. Introduction

Cells are endowed with a coordinated defense mechanism against lethal DNA damage, such as double-strand breaks (DSBs). Since mitosis with DSBs often has devastating consequences, entry into mitosis is blocked until completion of repair, usually by down-regulation of Cdk activity. Although the G2/M DNA damage checkpoint was originally proposed to be a devoted mechanism for such cell cycle delay, subsequent studies demonstrated its involvement also in other forms of damage response [1,2]. Recent proteomic analysis has revealed hundreds of phosphorylation targets of the checkpoint sensor kinases ATM and ATR with no apparent cell cycle connection, consistent with their direct roles in DNA repair [3].

Many observations have suggested that radiation sensitivity of AT cells which have mutations in the *ATM* gene is caused by a repair defect, not by a cell-cycle control defect [4–6], and studies at a molecular level have provided possible explanations for them. ATM is implicated in DSB end resection together with the Mre11–Rad50–Nbs1 complex, thus initiating both an ATR-dependent damage response and homologous recombination (HR) probably through phosphorylation of unknown targets [7]. ATM also phosphorylates Artemis proteins, affecting the other major DSB repair pathway non-homologous end-joining (NHEJ) [8]. While these results certainly established that the checkpoint machinery in higher eukaryotic cells is directly involved in DSB repair reaction [9], how its cell-cycle control role and repair role relatively contribute to overall repair efficiency and cellular survival has not been fully understood. In budding and fission yeasts, a role for the checkpoint machinery other than cell cycle delay has been extensively studied in the fork stabilization process during challenged DNA replication [10–12], but its significance in the G1 or G2 period is obscure. The ATR homolog Mec1 in budding yeast and Rad3 in fission yeast play a central role in triggering the damage checkpoint pathway. Bashkirov et al. have shown that both spontaneous and methyl methanesulfonate-induced allelic HR is severely compromised in *mec1* cells and that Mec1 phosphorylates Rad55 HR protein in a damage-dependent manner, although the connection between Rad55 phosphorylation and HR has not been

* Corresponding author at: Department of Tumor Biology, Institute of Biomedical Sciences, Iwate Medical University, 2-1-1 Nishitokuta, Yahaba-cho, Shiwa-gun, Iwate 028-3694, Japan. Tel.: +81 196515111x5631; fax: +81 196810025.
E-mail addresses: syasuhir@iwate-med.ac.jp (S. Yasuhira), ta-saito@rri.kyoto-u.ac.jp (T. Saito), chihaya@iwate-med.ac.jp (C. Maesawa), tmasuda@iwate-med.ac.jp (T. Masuda).

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addressed [13]. A similar assay designed to assess spontaneous and DSB-triggered HR in fission yeast failed to find any pronounced HR defect in *rad3* cells [14].

Most studies attempting to address the effect of the checkpoint machinery on HR using yeast systems have measured the number of recombination products or colony-forming ability. This type of end-point assay may have an intrinsic difficulty in excluding the effect of cell cycle modulation by the checkpoint machinery. In addition, reporter-based recombination assays inevitably measure recombinants arising from unequal crossing-over/gene conversion or allelic recombination events, which could be regulated differently from equal crossing-over or gene conversion between sister chromatids that may be predominant in HR repair after irradiation.

In the present study, we investigated how absence of checkpoint sensor kinase Rad3 or checkpoint effector kinases Chk1/Cds1 affects the DSB repair kinetics of *Schizosaccharomyces pombe* cells in G2 phase. To separate the repair defect from the cell cycle arrest defect, we employed two forced G2-arrest conditions. We found that *rhp51*-dependent repair kinetics was severely abrogated in checkpoint-deficient cells under these conditions, especially after high-dose irradiation. Intriguingly, formation of DSB-dependent foci by Rad11 (*S. pombe* Rpa1), Rad22 (*S. pombe* Rad52), or Rhp54 was not markedly affected by the checkpoint defect. We also demonstrated that Cds1 kinase can be activated and may have a role during G2 phase. Our results suggest that although the damage checkpoint machinery is not an absolute requirement for HR in G2 phase, it markedly affects the capacity of the latter.

2. Experimental procedures

2.1. Strains and media

Strains used in this study were constructed using standard techniques [15]. Genotypes and corresponding figure numbers are listed in Supplementary Table S1. Strains with the *cdc25-22* allele were grown in either YES or EMM at 25 °C, unless otherwise specified [15]. Those without the *cdc25-22* allele were cultured at 30 °C.

2.2. G2 synchronization

For *cdc25-22*-mediated G2 arrest, cells were grown in YES or EMM to 5×10^6/ml, transferred to a water bath at 36.5 °C or 35 °C (survival experiments), and cultured for 4 h. For sulfur-depletion-mediated G2 arrest, exponentially growing cells in EMM at 30 °C were washed three times with distilled water, re-suspended in EMM without Na_2SO_4 (EMM S-) at 3×10^6/ml, and cultured for 8 h. For septation index (SI) measurement, aliquots of cells were fixed in 70% ethanol and stained with DAPI for nuclei and calcofluor (Fluorescent Brightener 28, Sigma Chemical Co., Ltd.) for septa.

2.3. DSB induction

Cells were exposed to γ-ray radiation using a ^{60}Co γ-ray source (at the Research Reactor Institute, Kyoto University) at a dose rate of 6.7–67 Gy/min at room temperature. To minimize cell cycle progression during irradiation, irradiation time was kept shorter than 20 min by adjusting dose rate. For Zeocin (Invitrogen) treatment, G2-arrested *cdc25-22* cells were quickly collected by filtration through Whatman GF/A filters (GE Healthcare), resuspended in pre-warmed 10 mM phosphate buffer (pH 7.2) containing 10 μg/ml Zeocin (Invitrogen), and incubated at 36.5 °C for 30 min. The cells were then collected by filtration again, washed once with, and resuspended in, pre-warmed EMM, and cultured for repair at 36.5 °C.

2.4. PFGE and repair kinetics assay

Fifty million cells were collected at each time point, stopped quickly by adding sodium azide to a final concentration of 0.1%, and agarose plugs for PFGE were prepared as described previously [16,17]. Aliquots were fixed and kept aside for SI measurement. CHEF-DRIII (Bio-Rad) was employed for PFGE in 0.8% agarose gel with 1XTAE buffer. Electrophoresis was performed at 14 °C with a constant voltage gradient of 2 V/cm for 8 h at a 96° angle with a 20-min switching interval, for 8 h at a 102° angle with a 25-min switching interval, and for 8 h at a 108° angle with a 30-min switching interval [18]. The gels were stained with ethidium bromide and photographed. After densitometric scanning, the unrepaired fraction was calculated as the relative intensity of broken chromosomes in total DNA, and normalized against values obtained immediately after irradiation (100%) and non-irradiation (0%) (Supplementary Fig. S1).

2.5. γ-Ray sensitivity assay

Exponentially growing cells harboring the *cdc25-22* allele in YES or EMM liquid medium were diluted with the same medium to 1×10^4/ml and exposed to various doses of γ-ray radiation. With or without incubation at 35 °C for 4 h, 100 μl (1000 cells) were plated on a YES agar plate in duplicate. After incubation at 25 °C for 4 days, resulting colonies were counted.

2.6. Fluorescence microscopy

For analysis of radiation-induced foci, GFP- or CFP-tagged proteins expressed from the native promoters in living cells were observed using an AX-80 microscope (Olympus) with a 60× objective and appropriate filter sets. Images were captured with a DP-70 CCD camera (Olympus) and quantified for the numbers of foci per nucleus in at least 200 nuclei. For chromatid cohesion analysis, the *lacI-lacO* chromosome labeling system, originally developed in budding yeast [19], was used. The strain labeled at the *ade8* locus (FY15583) was obtained from the National Bioresource Project in Japan. Images were captured from living cells in eleven 0.5-μm-interval Z-sections, projected into a single image, and numbers of the GFP-LacI signals were counted. For cell cycle analysis using quantitative fluorescence microscopy, ethanol-fixed cells were treated with 100 μg/ml RNase A for 1 h and stained by resuspending the cell pellet in 25 mM sodium citrate, 18.75 μM Sytox Green (Invitrogen), 0.75 mM *p*-phenylenediamine and 37.5% glycerol. Cells were observed using a 20× objective. Regions with relatively uniform fluorescence intensity were cropped from the original images and processed using ImageJ. Data were pooled to yield at least 3000 nuclei for a single time point.

2.7. Cds1 kinase assay

Activity of Cds1 kinase was measured as described previously, with a slight modification [20]. Briefly, Cds1-HA was immunoprecipitated from the total cell lysate with anti-HA rat monoclonal antibody 3F10 (Roche Molecular Biochemicals) and Dynabeads Protein G (Invitrogen). Aliquots of the immunoprecipitants were analyzed for the amount of Cds1-HA by immunoblotting with anti-HA mouse monoclonal antibody 12CA5 (Roche Molecular Biochemicals). As a control, asynchronous or G2-presynchronized cells were cultured in the presence of 12 mM hydroxyurea (HU) for 2 h, and samples were prepared. The kinase reaction was performed in the presence of 10 μg/ml myelin basic protein (MBP, Wako Pure Chemical Industries Ltd.) and [γ-^{32}P]ATP (4500 Ci/mmol, MP Biomedical) at 30 °C for 15 min. The reaction was run through 15%