Mutation Research 791 (2016) 10–18

Contents lists available at ScienceDirect

Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis

journal homepage: www.elsevier.com/locate/molmut
Community address: www.elsevier.com/locate/mutres

PCNA SUMOylation protects against PCNA polyubiquitination-mediated, Rad59-dependent, spontaneous, intrachromosomal gene conversion

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ARTICLE INFO

Article history:
Received 15 January 2016
Received in revised form 29 June 2016
Accepted 1 August 2016
Available online 2 August 2016

Keywords:
Homologous recombination
Gene conversion
Siz1-dependent PCNA SUMOylation
Mms2/Rad5-dependent PCNA ubiquitination
S. cerevisiae

ABSTRACT

Homologous recombination is crucial in both the maintenance of genome stability and the generation of genetic diversity. Recently, multiple aspects of the recombination machinery functioning at arrested DNA replication forks have been established, yet the roles of diverse modifications of PCNA, the key platform organizing the replication complex, in intrachromosomal recombination have not been comprehensively elucidated. Here, we report how PCNA SUMOylation and/or polyubiquitination affects recombination between direct repeats in *S. cerevisiae*. Our results show that these PCNA modifications primarily affect gene conversion, whereas their effect on the recombination-mediated deletion of intervening sequence is much less obvious. Siz1-dependent PCNA SUMOylation strongly limits Rad52/Rad51/Rad59-dependent gene conversion. A 5- to 10-fold increase in the frequency of such recombination events is observed in Siz1-defective strains, but this increase is fully suppressed when PCNA polyubiquitination is also compromised. PCNA polyubiquitination can stimulate gene conversion in both PCNA SUMOylation-proficient and SUMOylation-deficient strains. On the other hand, in PCNA polyubiquitination-deficient strains, the lack of PCNA SUMOylation does not affect GC levels. Therefore, we postulate that the antirecombinogenic activity of Siz1 mainly concerns recombination induced by PCNA polyubiquitination. In the absence of PCNA SUMOylation, the frequency of PCNA polyubiquitination-mediated gene conversion is not only increased, but it is also channeled into the Rad59-dependent pathway. Additionally, we show a weak inhibitory effect of Rad5 on Rad52/Rad59-directed single-strand annealing.

1. Introduction

The complex process of faithful duplication of genetic material in S phase of the cell cycle is crucial for the maintenance of genetic stability. To avoid chromosomal rearrangements and point mutations, DNA replication must be facilitated by various mechanisms, among which DNA damage tolerance (DDT) and homologous recombination (HR) are involved in the repair of single-stranded gaps occurring in replicated DNA across template lesions or other obstacles blocking replication fork progression. DDT depends on the Rad6/Rad18-mediated ubiquitin conjugation system, which either directs translesion synthesis (TLS) polymerases to bypass damaged DNA in an error-free or error-prone manner [35,25,38] or activates error-free damage avoidance by Rad5-Ubc13-Mms2-mediated template switching (TS). In this process, the newly synthesized strand of the sister chromatid is transiently used as a template by the regular replicative polymerase [45,4]. Additionally, HR engages products of the *RAD52* epistasis group of genes to rescue stalled or collapsed replication forks by searching for and/or invading and copying information from a homologous template [50,17,41,28]. Based on genetic analysis, genes encoding enzymes involved in HR and DDT have been assigned to different epistatic groups. However, the relationship between the HR and DDT pathways seems to be more complex than was originally believed, as recent results suggest that recombination proteins are involved in the TS pathway [17,4,48,19,3].

The access of various repair machineries to a stalled replication fork is mainly regulated by the modifications of proliferating cell nuclear antigen (PCNA). Three molecules of PCNA form a replication processivity clamp, which also functions as a platform recruiting repair factors to DNA [33,11,21]. Monoubiquitination of Lys 164 in PCNA, mediated by the Rad6/Rad18 complex, stimulates TLS by enhancing the intrinsic affinity of TLS polymerases for PCNA through their ubiquitin-binding motifs [38]. On the other hand, the Ubc13/Mms2 ubiquitin-conjugating complex, acting in concert

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http://dx.doi.org/10.1016/j.mrfmmm.2016.08.001
0027-5107/

with the Rad5 ubiquitin ligase, decorates monoubiquitinated PCNA with a Lys 63-bound polyubiquitin chain, which in turn initiates TS. How PCNA polyubiquitination stimulates template switching remains enigmatic from a mechanistic point of view.

Consistent with the roles of TLS and TS in DNA damage tolerance, the level of PCNA mono- and polyubiquitination increases after exposure of eukaryotic cells to replication-blocking agents [10,13]. The enzymes responsible for ubiquitinating PCNA are also involved in the maintenance of genetic stability in untreated cells, as defects in Rad6, Rad18, Rad5, Mms2 and/or Ubc13 cause spontaneous mutator phenotypes [6,7,9,20].

In addition to ubiquitination, Lys 164 of PCNA can also be SUMOylated by the SUMO-conjugating enzyme Ubc9, which cooperates in this process with the SUMO ligase Siz1. The SUMOylation of PCNA occurs in S phase, independent of replication stress [22], and is believed to have an antirecombinogenic effect. Accordingly, PCNA SUMOylation defect caused by deletion of the *SIZ1* gene allows Rad52-dependent HR to suppress sensitivity to the lethal and mutagenic effects of UV in yeast defective in TS [36,37,15,20]. The DNA strand annealing activity of Rad52 plays a central role in the recombinogenic repair of DNA double-strand breaks (DSBs) and stalled or collapsed replication forks as well as in the repair of single-stranded DNA gaps and shortened telomeres. Based primarily on studies addressing double-strand break (DSB) repair, at least two major Rad52-dependent HR pathways have been discerned [23,26,31]. One requires the formation of a Rad51 filament on single-stranded DNA, which initiates invasion of DNA duplex by the Rad51 nucleofilament and D-loop formation. This recombination pathway is stimulated by Rad55, Rad57 and Rad54 accessory proteins and primarily leads to gene conversion (GC). The second pathway is independent of Rad51-mediated strand invasion; it relies on single-strand annealing (SSA) activity mediated by Rad52 and often also requires Rad59, which enhances the annealing activity of Rad52 [47,12]. Both Rad51-dependent and Rad51-independent pathways have also been identified among spontaneous recombination events.

In response to PCNA SUMOylation, Srs2 is recruited to replication sites. Srs2 is a UvrD-like helicase that can dismantle Rad51 presynaptic nucleofilaments and thereby prevent DNA strand invasion [27,49]. In addition, recent reports show that SUMOylated PCNA, acting in concert with Srs2, limits the synthesis-dependent extension of a recombination intermediate [8,30], pointing to an additional mechanism by which PCNA SUMOylation can affect HR. How the PCNA SUMOylation-mediated mechanisms affect different pathways of spontaneous intrachromosomal HR is not clear. Additionally, the complex relationships between HR and error-free DDT [5,32] give rise to the question of what the roles PCNA polyubiquitination and its interplay with PCNA SUMOylation are in recombination.

In the current study, we analyzed spontaneous recombination between direct repeats located over 5 kb apart to determine how Rad5 and Mms2 (responsible for PCNA polyubiquitination) and Siz1 (which mediates PCNA SUMOylation) affect Rad51-dependent intrachromosomal GC and Rad51-independent SSA in the yeast *S. cerevisiae*. We found that PCNA SUMOylation protects against non-canonical GC, which in addition to Rad52 and Rad51 is dependent on Rad59 and PCNA polyubiquitination.

2. Materials and methods

2.1. Yeast strains

The haploid *Saccharomyces cerevisiae* strains used to determine intrachromosomal recombination rates are listed in Table 1. All of the strains are derivatives of YWT-6 [*MAT*a *ade2-1(och) his3-11,15 leu2EcoRI:URA3:leu2-BstEII trp1-1(am) ura3-1 can1-100 RAD5*$^+$] and LSY1892 [*MATa ade2-n-URA3-ade2-a his3-11,15 leu2-3,112 trp1-1(am) can1-100 RAD5*$^+$], carrying the recombination systems described in Zheng et al. [51] and Fung et al. [16], respectively.

Targeted genes disruptions were performed *via* direct transformation of yeast cells with PCR-amplified disruption cassettes. *kanMX4* disruption cassettes were amplified through PCR using genomic DNA from appropriate BY4741 derivatives (Euroscarf) carrying deletions of ORFs of interest, with the corresponding primers A and D from the Saccharomyces Genome Deletion Project. The *rad5:HIS3* cassette was amplified *via* PCR as described previously [20]. Strains disrupted with *natMX4* or *hphMX4* cassettes were constructed by replacing the *kanMX4* marker in the BY4741-derived *kanMX4* strains (Euroscarf) with the *natMX4* or *hphMX4* marker according to the procedure of Goldstein and McCusker [18]. The desired integrants were verified through PCR and subsequent analysis of the respective DNA repair phenotypes.

2.2. Recombination assay

To study the recombination between the *leu2* repeats, yeast strains were first grown on plates containing synthetic complete (SC) medium without uracil (to ensure that the strains maintained the recombination reporter), then plated on YPD plates supplemented with 1% adenine (YPDA) and grown for 2–3 days to obtain single colonies. Single colonies from the YPDA plates were dispersed in 1 ml of sterile H_2O. The number of recombinants that occurred *via* SSA was estimated by plating 100 μl of the cell suspension (or an appropriate dilution) on SC plates supplemented with 5-fluoroorotic acid at 750 mg/l (5-FOA plates). GC events were detected by plating 100 μl of the cell suspension (or an appropriate dilution) on SC plates without uracil and leucine. To estimate the number of colony forming units (CFU), serial dilutions were plated on SC plates. The plates were incubated at 30 °C for 3 days before counting. The frequency of recombination was calculated as the ratio of Ura$^+$ Leu$^+$ revertans or FOA^R mutants to the number of CFU. The data from 20 to 100 independent cultures from 3 to 20 independent experiments were used for determination of the recombination frequency in each strain. At least two independently obtained strains of each genotype were employed in the assays. Confidence intervals (95%) and *P* values for the statistical differences in recombination frequencies between the analyzed strains were determined using *t*-test in the Statgraphics Centurion XVII program. The recombination between direct repeats of *ade2* alleles was investigated according to Fung et al. [16].

3. Results

3.1. Requirement of Rad52, Rad51 and Rad59 for spontaneous intrachromosomal recombination between direct repeats

To analyze the genetic requirements for spontaneous intrachromosomal recombination, we used the method described by Zheng et al. [51], employing haploid yeast strains carrying the *leu2-ecoRI:URA3:leu2-bstEII* recombination system on the left arm of chromosome III. In this system, direct 2.4 kb repeats of *leu2* alleles harboring different mutations are located 5.3 kb apart. Rad51-dependent GC between the repeats leads to a Leu$^+$ Ura$^+$ phenotype, whereas resistance to 5-FOA, resulting from the deletion of *URA3* (located in the intervening sequence between the *leu2* repeats) reflects Rad51-independent SSA events (Fig. 1).

As expected, based on the known role of Rad52 in recombination, in yeast proficient in both PCNA SUMOylation and ubiquitination, Rad52 deficiency caused a major decrease in the