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Unique and overlapping functions of the Exo1, Mre11 and Pso2 nucleases in DNA repair

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Abstract

The Mre11 and Pso2 nucleases function in homologous recombination and interstrand cross-link (ICL) repair pathways, respectively, while Exo1 nuclease is involved in homologous recombination and mismatch repair. Characterization of the sensitivity of single, double and triple mutants for these nucleases in *Saccharomyces cerevisiae* to various DNA damaging agents reveals complex interactions that depend on the type of DNA damage. The *pso2* mutant is uniquely sensitive to agents that generate ICLs and *mre11-H125N* shows the highest sensitivity of the single mutants to ionizing radiation and methyl methane sulfonate. However, elimination of all three nucleases confers higher sensitivity to IR than any of the single or double mutant combinations indicating a high degree of redundancy and versatility in the response to DNA damage. In response to ICL agents, double-strand breaks still form in the triple nuclease mutant indicating that none of these nucleases is responsible for unhooking cross-links.

Keywords

Exo1; Mre11; Pso2; Interstrand cross-links; Homologous recombination

1. Introduction

Nucleases play essential roles in most DNA repair mechanisms and their functions are often carried out by redundant activities. This is exemplified in the case of mismatch repair in *Escherichia coli* in which the excision step can be performed by any one of four redundant exonucleases [1]. The repair of DNA double-strand breaks (DSBs) by homologous recombination (HR) involves 5'-3' degradation of DNA ends to generate 3' single-stranded DNA (ssDNA), the substrate for Rad51 binding [2,3]. The Rad51-ssDNA complex promotes homology searching and strand exchange to initiate HR. The formation of ssDNA at break sites is considered to be an essential early step in HR, but the nucleases that participate in this process have not been clearly defined. In eukaryotes, the Mre11-Rad50-Xrs2 complex rapidly associates with DSBs, signals DNA damage and regulates the resection process [2,4-6]. The Mre11 subunit of the complex has an exonuclease activity that degrades with 3'-5' polarity, incompatible with the observed resection of 5' ends in vivo, however, the Mre11 endonuclease activity could potentially function in resection in association with a DNA helicase [7-10]. Analysis of separation of function alleles of *MRE11* that eliminate endo- and exonuclease activities, but maintain Mre11-Rad50-Xrs2 complex formation, have shown an essential role

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for the Mre11 nuclease in removal of Spo11 protein covalently bound to 5' ends of DSBs during meiosis [8,11]. However, the Mre11 nuclease is dispensable for resection of ends produced by the HO endonuclease, which produces "clean" ends with 3'-hydroxyl and 5'-phosphate groups, for telomere maintenance, non-homologous end joining (NHEJ) and checkpoint functions [8, 12]. In contrast, *mre11* null (*mre11Δ*) mutants show very high sensitivity to DNA damaging agents, failure to initiate meiotic recombination, delayed processing of HO-induced DSBs, short telomeres, checkpoint defects and greatly reduced NHEJ [2]. The *mre11Δ* and Mre11 nuclease-defective (e.g., *mre11-H125N*) mutants are equally defective in processing hairpin-capped DSBs in vivo consistent with biochemical studies showing cleavage of hairpin structures by the Mre11-Rad50 complex [13-15]. Because *mre11-H125N* mutants are only sensitive to very high doses of ionizing radiation we have previously suggested the Mre11 nuclease is required to process ends that have covalent adducts, such as 3' phosphoglycolate groups, covalently bound proteins or DNA hairpins, but not ends that lack covalent modifications [2,12]. Mre11 has also been shown to exhibit AP lyase activity in vitro raising the possibility of a secondary role for Mre11 in base excision repair [16]. The Sae2 protein appears to function in collaboration with the Mre11 complex in processing DNA ends and the *sae2Δ* mutant phenotype is similar to the phenotype of *mre11-H125N* strains [13,15,17-20]. Recent studies have shown a single-stranded endonuclease activity associated with Sae2 that is stimulated by Mre11-Rad50-Xrs2 for hairpin cleavage suggesting these activities collaborate in DSB processing [21]. Although *sae2* mutants exhibit delayed processing of HO-induced DSBs, the defect is not as severe as observed for *mre11Δ* strains [17].

The Exo1 nuclease was first characterized as a 5'-3' double-stranded DNA (dsDNA) exonuclease induced during meiosis [22]. Genetic studies have shown a minor role for Exo1 in mismatch repair and in the formation of deletions between long direct repeats [23-25]. Exo1 also functions in the error-free post-replication repair pathway, resection of uncapped telomeres, and in processing stalled replication forks [26-28]. Resection of an HO-induced break is slightly delayed in *exo1* strains, but this does not impair homologous recombination by gene conversion and consequently the *exo1* mutant is not sensitive to IR [12,18,23]. Over-expression of Exo1 suppresses the DNA repair defect of *mre11*, *rad50* or *xrs2* mutants, and partially overcomes the defect in resection of an HO-induced DSB. Furthermore, *exo1 mre11* double mutants exhibit a synthetic growth defect and are more sensitive to MMS and IR than *mre11* single mutants [29-33]. While these studies suggest that Exo1 and the Mre11 complex have redundant roles in resection, the *mre11-H125N exo1* double mutant shows higher IR resistance than the *mre11Δ* and no defect in DSB-induced gene conversion [32]. Thus if the Mre11 nuclease and Exo1 do participate in end resection, they must be redundant with at least one other activity.

Pso2/Snm1 is a member of the metallo-β-lactamase family of proteins and exhibits 5' exonuclease activity in vitro [34]. The *pso2* mutant was isolated on the basis of sensitivity to the interstrand cross-linking (ICL) agent psoralen, but does not exhibit sensitivity to UV, IR or MMS [35]. The *pso2* mutation is epistatic with mutations in NER genes, but the incision step of ICL repair and subsequent conversion to DSBs in replicating cells occur normally in *pso2* mutants [36,37]. The nuclease activity of Pso2 is required for ICL repair, but the substrate has not been clearly defined [34,38]. Although the *pso2* single mutant has no defect in the repair of IR-induced DNA damage, in this study we show Pso2 is required for repair in the absence of the Mre11 nuclease.

2. Materials and Methods

2.1. Media and growth conditions

Rich medium (YPD) and synthetic complete (SC) medium lacking the appropriate amino acid or nucleic acid base were prepared as described previously. For HO induction, cells were grown

in SC medium with 2% raffinose substituting glucose as a carbon source. HO was induced by addition of 2% galactose to the medium. Sporulation medium contained 1% potassium acetate and the appropriate amino acids or nucleic acid bases at 1/5 of the concentration used in SC medium. Where indicated, methyl methane sulfonate (MMS) or hydroxyurea (HU) was added to liquid YPD agar prior to pouring plates. Yeast cells were grown at 30°.

2.2. Yeast strains

All strains are in the W303 background with the corrected *RAD5* allele (Table 1). The *exo1* and *mre11-H125N* strains have been described previously [32,39]. The *pso2::His3MX6* allele was generated by PCR-mediated gene disruption and His⁺ transformants were screened by PCR and subsequently by sensitivity to cisplatin to confirm the disruption. Haploid strains were crossed and tetrads from the resulting sporulated diploids were dissected and characterized by genotype and PCR to identify haploid segregants with the appropriate combinations of mutations.

2.3. Ionizing radiation, MMS, HU and mitomycin survival assays

For γ -ray (IR) sensitivity assays, cells were grown to exponential phase and aliquots of serial dilutions spotted onto solid YPD medium. The plates were irradiated in a Gammacell-220 containing ⁶⁰Co (Atomic Energy of Canada) for the designated dose. For cisplatin survival assays, cells were grown to exponential phase, cisplatin was added to the cultures to a final concentration of 500 μ M and growth continued for two hours. The cells were harvested, suspended in water and aliquots of serial dilutions spotted onto solid YPD medium. For HU and MMS sensitivity tests, the cells were grown as above and aliquots of serial dilutions spotted onto solid YPD medium containing HU or MMS. The plates were incubated for 3 days before being photographed. Each strain was assayed at least three times.

2.4. Physical analysis of mating-type switching

Physical analysis of mating-type switching was performed as described previously [8]. For genetic analysis of mating-type switching, cells were grown in raffinose medium to mid-log phase, HO endonuclease was induced for 2-hr and aliquots of diluted cultures were plated on solid YPD medium. The mating type of 100 independent colonies from each strain was determined by mating to *MATa* and *MAT α* tester strains.

2.5. Physical analysis of DNA intermediates in interstrand crosslink repair

PFGE analysis of DNA intermediates resulting from treatment with nitrogen mustard (HN2) was performed as described previously using doses of 10 or 100 μ M HN2 [40].

3. Results and discussion

3.1. Additive effects of *exo1*, *mre11-H125N* and *pso2* mutations on γ -ray sensitivity

Genetic analysis of yeast strains lacking the Mre11 nuclease activity (e.g. *mre11-H125N*) revealed weak sensitivity to ionizing radiation and no defects in processing HO-induced DSBs, telomere maintenance or end-joining repair [8]. This observation led to the idea that redundant nucleases in mitotic cells can process ends in the absence of the Mre11 nuclease. We have previously shown that while mutation of *EXO1* reduces the IR resistance of the *mre11-H125N* strain, the *exo1 mre11-H125N* double mutant is much more resistant to radiation than *mre11 Δ* strains [32] (Fig. 1). The Pso2 protein was recently shown to possess 5'-3' exonuclease activity [34], and therefore we tested the effect of deleting *PSO2* on the sensitivity of *exo1* and *mre11-H125N* strains to IR. As described previously, the *mre11-H125N* strain is resistant to 400 Gy IR, but plating efficiency is reduced 100-fold at 800 Gy; the *exo1* and *pso2* mutants show equivalent survival to wild type at both doses (Fig. 1). The IR sensitivity of the *mre11-*

H125N pso2 double mutant was increased by 10-fold compared with the *mre11-H125N* single mutant and was more sensitive to IR than the *mre11-H125N exo1* double mutant. The *exo1 pso2* double mutant was slightly more sensitive to IR than the single mutants, consistent with a previous report [36]. However, the *exo1 mre11-H125N pso2* triple mutant was more sensitive to IR than any of the double mutants, showing a >1000-fold decrease in cell survival at 800 Gy. Even though the triple mutant is sensitive to high doses of IR, the survival at 400 Gy is higher than the *mre11A* strain (Fig. 1).

To determine whether the *exo1 mre11-H125N pso2* triple mutant has increased sensitivity to other DNA damaging agents, all of the mutant combinations were assayed for sensitivity to cisplatin or MMS. As shown previously, the *pso2* single mutant is sensitive to cisplatin, which induces interstrand cross-links (Fig. 1). The *exo1* and *mre11-H125N* single mutants showed no sensitivity to 500 μ M cisplatin, but *mre11-H125N* caused a slight increase in the sensitivity of the *pso2* mutant. In contrast to IR, the *exo1 mre11-H125N pso2* showed no greater sensitivity to cisplatin than any of the *pso2* double mutants. It has previously been reported that *exo1 pso2* mutants show higher sensitivity to ICLs than *pso2* single mutants [36], but this phenotype was not seen under the conditions used here. In the previous study, the strains used contain the *rad5-535* hypomorphic allele and this could have contributed to the increased sensitivity to crosslinking agents noted for the *exo1 pso2* strain because we found the *rad5-535* allele to increase the MMS and cisplatin sensitivity of the *exo1 mre11-H125N pso2* strain compared with the isogenic *RAD5* version (data not shown).

At 0.01% and 0.02% MMS all of the strains showed high survival, but the colony size of the *exo1 mre11-H125N* double mutant was reduced compared with cells grown on YPD, this was most noticeable after 2 days growth (data not shown). At 0.03% MMS, the *mre11-H125N* single mutant showed 10-fold reduced plating efficiency and the *exo1 mre11-H125N* double mutant exhibited a further 10-fold decrease in survival and small colony size (Fig. 1). Even at 0.03% MMS, the *pso2* strain showed normal survival and did not synergize with *exo1* or *mre11-H125N*. Surprisingly, the *pso2* mutation partially suppressed the MMS sensitivity of the *mre11-H125N* single and *exo1 mre11-H125N* double mutants. This slight suppression suggests Pso2 acts in a negative way on substrates or intermediates in the repair of MMS-induced damage that accumulate in the *mre11-H125N* mutant. Because treatment of cells with MMS results in replication fork stalling, the mutants were also tested for sensitivity to hydroxyurea, which stalls replication forks by decreasing nucleotide pools instead of damaging the DNA template. At 100 mM HU, the *mre11-H125N exo1* double mutant showed a 10-fold reduction in plating efficiency (Fig. 1). This could be due a slight defect in the S-phase checkpoint or due to inefficient repair of collapsed replication forks. As observed for MMS, the *pso2* mutation partially suppressed the HU sensitivity of the *mre11-H125N exo1* double mutant. The different behavior of the *pso2* mutation in combination with *mre11-H125N* in response to MMS and HU, compared with IR and cisplatin, could be due to the addition of the chemical to the growth medium requiring that the mutants grow with continual low-level damage. Formation of a colony requires multiple cell divisions and therefore growth of mutants with a subtle defect in the checkpoint response may be particularly sensitive to continual low-level damage. In the case of IR and cisplatin, the cells are exposed to single high dose.

3.2. DSBs are induced by nitrogen mustard in the *pso2* mutants

Repair of ICLs in exponential cultures requires genes of the *RAD3* (nucleotide excision repair) and *RAD52* (homologous recombination) epistasis groups and involves a DSB intermediate [40]. DSBs are thought to arise when a replication fork encounters a nick in the template strand due to incision at the crosslink. Previous studies have shown that ICL-induced DSBs still form in *rad1*, *rad2* and *exo1 pso2* mutants suggesting these nucleases are not responsible for the incision event at ICLs [36,40]. To determine whether DSBs are formed after treatment with

an ICL agent in the *mre11-H125N pso2* and *exo1 mre11-H125N pso2* mutants, cultures were grown to exponential phase, treated with HN2 for 2 hrs and then chromosomal DNA was analyzed by PFGE (Fig. 2). DSBs were readily observed, even with 10 μ M HN2, indicating that the defect in ICL repair is not at the incision step and is due to a defect in processing the DSB intermediate formed. The increase in low molecular weight DNA products in the *pso2* strains compared with wild type is presumably due to the greatly reduced repair capacity of these strains.

3.3. The *exo1 mre11-H125N pso2* mutant shows normal processing of HO-induced breaks and gene conversion

To determine whether the sensitivity of the *exo1 mre11-H125N pso2* triple mutant to IR is due to decreased efficiency of DSB repair a mating-type (*MAT*) switching assay was performed [3]. The repair of an HO-induced DSB was monitored at the DNA level after induction of the HO endonuclease for one hour. To measure the formation of recombinant products the DNA samples were digested with *StyI*, which cuts within $Y\alpha$ but not $Y\alpha$ sequences (Fig. 3A). HO cleavage produces a 0.7-kb cut fragment that disappears over time concomitant with repair to $MAT\alpha$ yielding a 1.8-kb fragment. The kinetics of *MAT* switching was similar to the wild type strain suggesting no defect in processing the HO-induced break in the *exo1 mre11-H125N pso2* triple mutant (Fig. 3A). In *mre11 Δ* strains the HO cut fragment persists for longer and repaired products are delayed by one hour [8,41]. The number of cells that switched mating type after inducing HO for two hours was also scored genetically and no significant difference was observed between the *exo1 mre11-H125N pso2* triple mutant and wild type (data not shown). To look specifically at DSB processing, HO was induced in strains containing a *rad52* mutation to prevent repair of the break fragment by homologous recombinational repair. In the *rad52* mutant, the 0.7-kb HO break fragment disappears after several hours due to 5'-3' resection of the DSB past the *StyI* site rendering the ssDNA region resistant to *StyI* digestion (Fig. 3B). Null mutations in *MRE11* reduce processing of DSBs leading to persistence of the 0.7-kb HO break fragment for several hours in the *rad52* background. In the *rad52 exo1 mre11-H125N pso2* mutant the disappearance of the 0.7-kb HO break fragment showed the same kinetics as the *rad52* single mutant suggesting 5'-3' resection of the DSB is normal. Thus the sensitivity of the *exo1 mre11-H125N pso2* triple mutant to IR appears to be unrelated to nuclease resection of "clean" DSBs. We suggest the high sensitivity of the triple mutant to IR is due to defective processing of DNA ends that might have covalent adducts attached, or to interstrand cross-links induced by IR.

3.4. Telomere length is normal in the *exo1 mre11-H125N pso2* triple mutant

Mammalian cells encode three proteins related to Pso2: Snm1A, Snm1B (Apollo) and Snm1C (Artemis). Apollo was recently identified as a TRF2-interacting protein and localizes to telomeres in mouse and human cells [42,43]. To determine whether the *pso2* mutation affects telomere maintenance total genomic DNA was isolated from mutants and digested with *XhoI*, which cuts within the Y' sub-telomeric repeats to liberate a terminal fragment of about 1.5-kb [8]. As shown previously, the *exo1* and *mre11-H125N* strains exhibit normal telomere length. The *pso2* mutation has no obvious effect on telomere length, either by itself or in combination with *exo1* and/or *mre11-H125N* (Fig. 4). The Mre11-Rad50-Xrs2 complex is required for recruitment of telomerase and, consequently, *mre11 Δ* mutants have shorter telomeres than wild type [44,45]. The *mre11 Δ* strain was used as a control for a mutant with short telomeres.

In summary, we show that the Mre11, Exo1 and Pso2 nucleases contribute to the repair of the IR-induced DNA damage. The role of Pso2 in repair is only revealed in the absence of the Mre11 nuclease suggesting these two nucleases have common substrates. Processing of an HO endonuclease-induced DSB still occurs in the *exo1 mre11-H125N pso2* triple mutant indicating these nucleases do not have a significant role in 5'-3' resection of "clean" ends and the IR

sensitivity is most likely due to processing other IR-induced lesions, such as covalent adducts and cross-links, prior to extensive resection. Recent studies suggest Exo1 and Sae2 have redundant roles in processing HO-induced DSBs [18]. We found the *sae2* mutation to behave similarly to *mre11-H125N* in combination with *exo1* or *pso2* (A. Lam, E. Mimitou and L. Symington, unpublished results). The sensitivity of the *pso2* mutant to cisplatin is slightly increased by the *mre11-H125N* mutation indicating one shared substrate could be interstrand cross-links. The MMS and HU sensitivity of the *mre11-H125N* and *mre11-H125N exo1* mutants is partially suppressed by *pso2* indicating the complex interactions between these mutations. The suppression of MMS sensitivity could be due accumulation of a repair substrate or intermediate in the *mre11-H125N* that is converted to a toxic lesion by the Pso2 nuclease. Alternatively, Pso2 may suppress checkpoint signaling in the presence of continuous low-level DNA damage or replication fork stalling.

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Figure 1.

Sensitivity of nuclease mutants to DNA damaging agents. Ten-fold serial dilutions of each strain were spotted onto YPD and left unirradiated or irradiated with 400 or 800 Gy, or spotted onto YPD containing the indicated amount of MMS or HU. Strains were treated with 500 μ M cisplatin for 2 hr and then ten-fold serial dilutions were spotted onto YPD.

Figure 2.

The *pso2* mutants retain the ability to convert ICLs to DSBs. Exponential-phase cultures were treated with HN2 for 2 hr, harvested, washed, and grown in SC for 1 hr, and conversion of DNA to DSBs assayed by PFGE.

Figure 3.

DSB-resection and mating-type switching are normal in the *exo1 mre11-H125N pso2* triple mutant. A. The left panel shows a schematic representation of the *MATa* locus, *StyI* sites and the expected fragments before and after HO cleavage are indicated. Repair of the HO-induced break by conversion from the *HMLα* donor yields a novel 1.8-kb *StyI* fragment. Time course of repair in wild type and *exo1 mre11-H125N pso2* strains is shown in the right panel. B. 5'-3' resection from the HO cut site past the *StyI* site renders the site resistant to cleavage by *StyI* resulting in disappearance of the 0.7-kb fragment. Time course of HO cut fragment stability in *rad52*, *rad52 mre11* and *rad52 exo1 mre11-H125N pso2* strains. All strains contain the pGAL-HO plasmid. Galactose was added to the cultures at time 0, for 1 hr, to induce expression of HO endonuclease and samples removed at the indicated time interval for DNA analysis.

Figure 4.

Telomere length is unaffected by the *pso2* mutation. Genomic DNA isolated from each strain was digested with *Xho*I, separated on a 1% agarose gel, transferred to a nylon membrane and hybridized with a telomere-specific probe. The Y' telomeres form a heterogenous band of ~1.5-kb.

Table 1

Yeast strains

Strain number	Genotype	Source or reference
W1588-4A	<i>MATa</i>	R. Rothstein
W1588-4C	<i>MATa</i>	R. Rothstein
LSY624	<i>MATa</i> <i>exo1::URA3</i>	[39]
LSY721	<i>MATa</i> <i>rad52::LEU2 met17::ADE2</i>	[46]
LSY781	<i>MATa</i> <i>mre11-H125N::URA3::mre11-H125N</i>	[8]
LSY872-2B	<i>MATa</i> <i>met17-sna ADE2 mre11-H125N::URA3::mre11-H125N</i>	[47]
LSY927-4A	<i>MATa</i> <i>exo1::HIS3 met17::ADE2</i>	[47]
LSY1397	<i>MATa</i> <i>mre11-H125N</i>	[11]
LSY1419	<i>MATa</i> <i>exo1::HIS3 mre11-H125N::URA3::mre11-H125N</i>	This study
LSY1631	<i>MATa</i> <i>mre11-H125N::URA3::mre11-H125N pso2::His3MX6</i>	This study
LSY1639	<i>MATa</i> <i>pso2::His3MX6</i>	This study
LSY1640	<i>MATa</i> <i>pso2::His3MX6</i>	This study
LSY1724-29A	<i>MATa</i> <i>exo1::HIS3 mre11-H125N::URA3::mre11-H125N pso2::His3MX6 rad52::LEU2</i>	This study
LSY1760-3D	<i>MATa</i> <i>exo1::URA3 pso2::His3MX6</i>	This study
LSY1992-16C	<i>MATa</i> <i>exo1::URA3 mre11-H125N::URA3::mre11-H125N pso2::His3MX6</i>	This study

All strains are in the W303 background (*leu2-3,112 trp1-1 ura3-1 can1-100 ade2-1 his3-11,15 RAD5*).