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Pso2 (SNM1) is a DNA structure-specific endonuclease

Tracy Tiefenbach[1] and Murray Junop[1,2,*]

[1]Department of Biochemistry and Biomedical Sciences and [2]M.G. DeGroote Institute for Infectious Disease Research, McMaster University, Hamilton, Ontario L8N 3Z5, Canada

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ABSTRACT

Many types of DNA structures are generated in response to DNA damage, repair and recombination that require processing via specialized nucleases. DNA hairpins represent one such class of structures formed during V(D)J recombination, palindrome extrusion, DNA transposition and some types of double-strand breaks. Here we present biochemical and genetic evidence to suggest that Pso2 is a robust DNA hairpin opening nuclease in budding yeast. Pso2 (SNM1A in mammals) belongs to a small group of proteins thought to function predominantly during interstrand crosslink (ICL) repair. In this study, we characterized the nuclease activity of Pso2 toward a variety of DNA substrates. Unexpectedly, Pso2 was found to be an efficient, structure-specific DNA hairpin opening endonuclease. This activity was further shown to be required *in vivo* for repair of chromosomal breaks harboring closed hairpin ends. These findings provide the first evidence that Pso2 may function outside ICL repair and open the possibility that Pso2 may function at least in part during ICL repair by processing DNA intermediates including DNA hairpins or hairpin-like structures.

INTRODUCTION

DNA hairpins pose a significant challenge to genome stability. Once formed, such structures must be removed and/or opened by specialized nucleases to permit subsequent repair. DNA hairpins may be generated in a variety of ways with perhaps the most well characterized being programmed hairpin formation resulting from V(D)J recombination (1,2). Hairpin structures capping DNA ends are also formed at inverted repeat sequences and in some instances, as intermediates during DNA repair (3,4). Regardless of how hairpins are generated, these ends must be processed appropriately before ligation can occur. Failure to do so results in accumulation of double-strand breaks (DSBs), and thus a potentially lethal cellular event [reviewed in (4,5)].

Cells maintain at least two mechanisms for removal of DNA hairpins (Supplementary Figure S1). The first involves the Mre11/Rad50/Xrs2(Nbs) MRX (MRN) complex which is well characterized for its essential role in processing DSB during repair (6,7). This complex has 3′-exonuclease activity necessary for DSB repair as well as a structure-specific endonuclease hairpin opening activity important for stability of inverted repeat sequences (3,8). In conjunction with Sae2, MRX has been shown to catalyze the entire removal of DNA hairpin structures. MRX/Sae2 functions at damaged cruciform structures that have been converted into DNA hairpins with a single strand nick distal to the hairpin cap (9,10). MRX is able to open a gap from the available nick using its 3′-exonuclease activity and stimulates Sae2 to cleave the resulting ssDNA on the opposing strand, thereby completely removing the hairpin structure. Artemis (SNM1C) represents a second specialized nuclease capable of processing DNA hairpins; however, its function appears to be largely restricted to repair associated with the non-homologous end-joining (NHEJ) DSB repair pathway following V(D)J recombination. The structure-specific endonuclease activity of Artemis (SNM1C) generates nicks at or very close to the apex of DNA hairpin structures, generating free ends for further processing and repair (11). A similar activity has also been reported for the MRX/Sae2 complex in yeast (9,12).

Artemis belongs to a larger family of nucleases that share a conserved β-CASP domain essential for catalytic activity (13–16). Members of the β-CASP family include SNM1A (Pso2 in yeast), SNM1B (Apollo), SNM1C (Artemis), CPSF-73 and ELAC2. ELAC2 and CPSF-73 are involved in processing of tRNA and mRNA, respectively (17,18). While SNM1A and B exhibit 5′ exonuclease activity, only SNM1C (Artemis) has been shown to harbour endonuclease activity capable of opening DNA

*To whom correspondence should be addressed. Tel: +905 525 9140 (Ext 22912); Fax: +905 522 9033; Email: junopm@mcmaster.ca

hairpins. SNM1A (Pso2 in yeast) and SNM1B (Apollo) function at different steps in ICL repair, with Apollo acting prior to DSB formation and SNM1A (Pso2 in yeast) functioning at some point subsequent to DSB formation (19,20). In fact, Apollo is required for generating DSBs at stalled replication forks subsequent to ICL exposure (21). Given the close phylogenetic relationship between Pso2 and SNM1C (Artemis) it has been suggested that Pso2 may function in repair of ICL lesions through cleavage of hairpin-like structures (22).

Despite having an important role in ICL repair, very little is currently known about how Pso2 functions inside the cell. *PSO2* (*SNM1*) was first identified in a screen of yeast mutations that greatly increased sensitivity to the crosslinking agent, **pso**ralen (23). *SNM1* was later found to be allelic to *PSO2,* which when deleted rendered cells **s**ensitive to **n**itrogen **m**ustard, a crosslinking agent (24,25). Interestingly Pso2 does not appear to function in DNA repair pathways required for ICL repair such as homologous recombination and translesional synthesis. Mutations in *PSO2* result in an accumulation of DNA DSBs following exposure to ICL inducing agents suggesting that Pso2 acts downstream of the incision event in ICL repair (26). Cells harbouring a *pso2* deletion exhibit no significant sensitivity to other DNA damaging agents including ionizing radiation, UVC exposure and HO endonuclease, suggesting a specialized role for Pso2 in the repair of DSBs formed during repair of ICL lesions (23,26–28). The specific requirement of Pso2 in ICL repair suggests that these breaks may be in some way different from other forms of DNA breaks.

The question then becomes what DNA intermediate is generated during ICL repair that Pso2 is uniquely able to act upon? To begin addressing this question, we have analyzed the activity of Pso2 towards several DNA intermediates that may be encountered during ICL repair. Interestingly, Pso2 was found to possess an efficient endonuclease activity specific to DNA hairpin structures. We further demonstrate the requirement of this activity *in vivo* for restoring genomic DNA when DSBs are generated containing covalently closed DNA hairpins. Thus, yeast Pso2 appears to retain two distinct nuclease activities, an exonuclease activity able to degrade DNA from a free 5′-end and an endonuclease activity specific to DNA hairpin structures. These findings suggest that Pso2 may also function outside of ICL repair, in particular DNA hairpin repair and suggest that Pso2 and hSNM1A may function at least in part during ICL repair by processing DNA intermediates including DNA hairpins and/or hairpin-like structures, generated directly or indirectly by ICL damage.

MATERIALS AND METHODS

Plasmid construction, expression and purification of Pso2

The *Saccharomyces cerevisiae PSO2* gene from pQE32 (generously provided by James Hejna, Department of Molecular and Medical Genetics, Oregon Health and Sciences University) was amplified by PCR and cloned into the pDEST14 expression vector using Gateway cloning technology (Invitrogen). The *Escherichia coli* cell line Rossetta (DE3) pLysS (Novagen), harbouring the *PSO2* expression vector, was grown at 37°C prior to induction with 1 mM IPTG at 16°C overnight. Following induction, cells were harvested by centrifugation at $10\,000 \times g$ for 15 min, washed once with phosphate buffered saline (PBS) and re-suspended in lysis buffer (50 mM sodium phosphate pH 7.0, 500 mM NaCl, 3 mM β-mercaptoethanol, 1% Triton X-100). Cells were lysed by four sequential passages through a French Press cell pressurized to 12 000 pounds per square inch. Lysate was clarified via centrifugation at $50\,000 \times g$ for 40 min prior to injecting sample onto a 5 ml, Ni^{2+}charged HiTrap Chelating column (Amersham) pre-equilibrated with buffer A (50 mM sodium phosphate pH 7.0 and 500 mM NaCl). Subsequent washes with buffer A supplemented with 15, 30 and 45 mM imidazole occurred before step elution with buffer A containing 210 mM imidazole. Dithiothreitol (DTT) and ethylenediaminetetraacetic acid (EDTA) were immediately added to the eluate to a final concentration of 5 and 1 mM, respectively. The sample was then diluted to a final concentration of 100 mM NaCl using Buffer B (50 mM sodium phosphate pH 7.0, 1 mM EDTA and 5 mM DTT) and injected onto a 5 ml HiTrap Q sepharose column (Amersham) equilibrated with buffer B with 100 mM NaCl. Pso2 was eluted in a gradient from 100 to 200 mM NaCl. Final protein was exchanged into buffer containing 10 mM Tris pH 7.0, 100 mM NaCl and 5 mM DTT prior to concentrating by ultracentrifugation (30 MWCO, macrosep). Samples were frozen at −80°C in 10% (v/v) glycerol. The *pso2* mutant H611A was created using site directed mutagenesis (QuikChange, Stratagene) and purified as wild-type.

Pso2 was quantified using absorbance measured at 280 nm after dilution into 6 M guanidine–HCl by the method of Pace *et al.* (29). Western blot analysis was performed according to the manufactures instructions (Perkin Elmer) with primary detection using mouse anti-His antibody followed by identification with secondary goat anti-mouse antibody conjugated with horse radish peroxidase.

DNA substrates

Oligonucleotides labelled at the 5′-end were generated with [γ-^{32}P]-ATP (Perkin Elmer) using T_4 polynucleotide kinase according to manufacturer's instructions (New England BioLabs). Oligonucleotides labelled at the 3′-end were generated using Klenow exo$^-$ and (α-^{32}P)-GTP (Perkin Elmer) according to manufacturer's instructions (New England Biolabs). Labelled oligonucleotides were purified using denaturing polyacrylamide (10%) gel electrophoresis (PAGE). Double stranded substrates were generated by annealing labelled oligonucleotide with a molar equivalent of the non-labelled complementary oligonucleotide to produce blunt ends, 3′-overhangs and 5′-overhangs. Annealing was performed by heating to 95°C for 5 min followed by cooling to room temperature over 60 min. Hairpin substrates were annealed at 1 μM concentrations to promote intermolecular interactions. Treatment with T7 exonuclease and RecJf verified the

integrity of the substrates. All of the DNA substrates labelled at the 3′-end contained a free 5′-phosphate unless stated otherwise. Specific oligonucleotide sequences used in substrate generation are listed in Supplementary Table S1.

Double stranded DNA substrate containing two hairpin capped ends was generated by 5′-labelling of the sequence 5′-CAATCAAGGGAACCTTGATTGCAGAGATGGG AACCATCTCTG, prior to ligation with T4 ligase in the presence of 40 mM Tris–HCl pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP at 25°C overnight.

DNA substrate containing an interstrand crosslink was prepared using the method of K. Chválová *et al.* (30), by treating cisplatin (CAS 15663-21-1) with an equimolar concentration of $AgNO_3$ and removing the precipitate via centrifugation. Four molar equivalents of activated cisplatin were incubated with 1 M equivalent of the oligonucleotide 5′-CTTCCTTCTCCCTTCTCCTACTCTCCC TTCTCCCTCGCTCT, containing a 3′-label and 5′-phosphate, in 100 mM sodium perchlorate pH 5.6 at 37°C for 7 days in the dark. The oligonucleotide containing a monoadduct at G was gel purified and annealed with the complementary strand in the presence of 400 mM NaCl. The dsDNA was exchanged into 100 mM sodium perchlorate pH 5.6 and incubated at 37°C for 14 days in the dark. Quantification of DNA was performed by reading absorbance at 260 nm.

In vitro nuclease activity

Nuclease assays were performed by incubating varying concentrations of Pso2 with 1 pmol DNA in the presence of 10 mM Tris pH 7.9, 10 mM $MgCl_2$, 50 mM NaCl and 1 mM DTT in a final volume of 10 µl. Reactions were carried out at 25°C for various time intervals (0.25, 0.5, 0.75, 1, 2, 4, 8, 16 and 32 min) and quenched by addition of an equal volume of formamide and incubation at 90°C for 5 min. The DNA was resolved using 20% denaturing PAGE and analyzed by autoradiography.

Relative activities were determined using 5′-labelled substrates containing a non-bridging phosphorothioate substitution between the second and third nucleotide so that only a single reaction product could be generated for each DNA substrate. Relative activities for hairpin opening were calculated by quantifying the disappearance of substrate. Radiolabelled DNA substrates and products were quantified using ImageQuant (Molecular Dynamics). All experiments were performed in triplicate.

DNA-binding assays

Increasing amounts of Pso2 were added to 4 pmol of 3′-^{32}P end-labelled ssDNA containing a phosphorothioate substitution between the first and second nucleotide and either a 5′-phosphate or 5′-hydroxyl. Pso2 is unable to digest DNA substrates containing a phosphorothioate substitution at the non-bridging oxygen located between the first and second nucleotides (Supplementary Figure S2). Therefore, in order to analyze binding without the complication of substrate degradation, binding assays were performed using 5′-hydroxyl or 5′-phosphate containing substrates harbouring a phosphorothioate substitution after the first nucleotide. The reactions were carried out in the presence of 10 mM Tris pH 7.9, 50 mM NaCl, 1 mM DTT and 15% glycerol at 25°C for 20 min. The extent of DNA binding was analyzed by running reactions on an 8% non-denaturing gel in TAE buffer. Quantification of bound and unbound species was performed with ImageQuant (Molecular Dynamics).

In vivo endonuclease activity

Saccharomyces cerevisiae MYO37 was generously provided by Cliff Weil (Department of Agronomy, Purdue University) and transformed with pWL201 (31). These cells were grown to saturation at 30°C in drop out base media containing raffinose as a carbon source. Induction of transposase was carried out by the addition of 1% galactose. Cells were serially diluted and plated onto media lacking either uracil or both uracil and adenine. Transposition frequency was determined by dividing the number of ade$^+$ revertants by the total number of colony forming units.

RESULTS

Pso2 exonuclease activity is not dependant on DNA structure but does require a 5′-phosphate

Nuclease activity of highly purified Pso2 (Supplementary Figure S3) was analyzed using a variety of DNA substrates. In agreement with work by Li *et al.* (32), Pso2 was found to possess 5′- but not 3′-exonuclease activity (Figure 1). Our analysis further demonstrates that Pso2 is able to fully degrade the entire DNA substrate to a single mononucleotide indicating that Pso2 does not have a minimum DNA length requirement for activity.

An interesting observation made while analyzing the exonuclease activity of Pso2 was that only DNA containing a free 5′-phosphate could be degraded by Pso2. As shown in Figure 1, Pso2 fully degraded substrate containing a 5′-phosphate (Figure 1F) but not the identical substrate containing a 5′-hydroxyl (Figure 1E). Inability of Pso2 to degrade a 5′-hydroxyl containing substrate was not due to reduced substrate binding affinity (Figure 1G) as comparison of dissociation constants with 5′-OH and 5′-PO4 containing substrates (8.0 ± 0.6 and 9.0 ± 4.0 nM, respectively) showed no significant differences.

As shown in Supplementary Figure S4A–C, all double stranded DNA substrates (blunt ends, 3′- and 5′-overhangs) were efficiently digested from the 5′-end. However, if a 5′-OH was present, these DNA substrates were not degraded even when Pso2 was present at a 10-fold higher concentration (Supplementary Figure S4D and E). Importantly, these results clearly demonstrate that Pso2 does not retain endonuclease activity towards overhang structures (3′ or 5′), ssDNA or dsDNA substrates.

The unusual requirement of Pso2 exonuclease activity for a 5′-phosphate may be a feature conserved amongst β-CASP family members as similar requirements have also been reported for other members of this family including

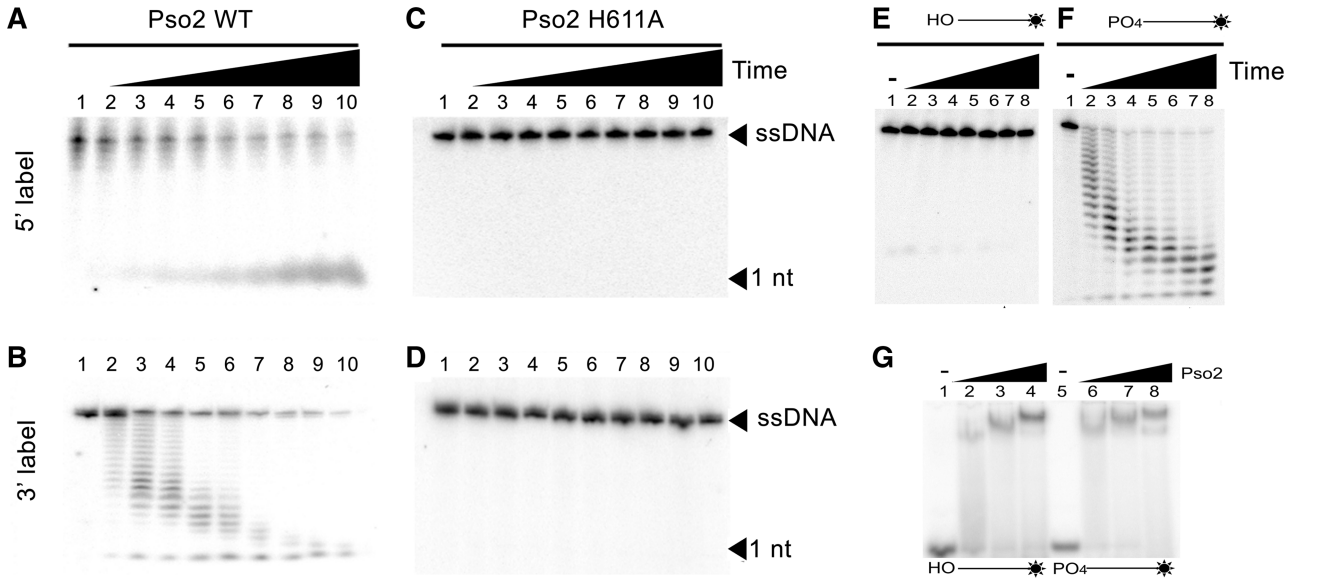

Figure 1. Pso2 possesses 5′ but not 3′-exonuclease activity and requires a 5′-phosphate for nucleotide removal. Pso2 (80 nM) was incubated with ssDNA (100 nM) labelled at the 5′ (**A**) or 3′-end (**B**). Purified *pso2* H611A, a catalytic mutant (1 μM) was assayed with ssDNA (100 nM) labelled at the 5′ (**C**) or 3′-end (**D**). Lane 1 in each panel contains no Pso2. Reactions in lanes 2–10 were carried out for increasing time intervals: 0.25, 0.5, 0.75, 1, 2, 4, 8, 16 and 32 min, respectively. In panel (**E**) Pso2 (1.5 μM) was assayed using 3′-labelled 20 nt ssDNA (200 nM) containing a 5′-hydroxyl. Lane 1 is a negative control in the absence of Pso2. Reactions in lanes 2–8 were incubated for 0.5, 1, 2, 3, 4, 8 and 16 min, respectively. Panel (**F**) is identical to panel (E) except substrate used contains a 5′-phosphate. (**G**) EMSA reactions using increasing amounts of Pso2 were incubated with a 3′-labelled 12 nt single-stranded DNA substrate (100 nM) containing a phosphorothioate substitution between the first and second nucleotide as well as either a 5′-phosphate or a 5′-hydroxyl. Lane 1 and 5, no Pso2 added; lanes 2–4 contain 5′-hydroxyl substrate with final Pso2 concentrations of 50, 100 and 200 nM, respectively. Lanes 6–8, 5′-hydroxyl substrate with final Pso2 concentrations of 50, 100 and 200 nM, respectively.

hSNM1A and hSNM1C/Artemis (11,33). The functional significance of this 5′-phosphate-dependence is not clear, however it may help to regulate exonuclease activity perhaps preventing unwarranted degradation at some single-stranded DNA nicks generated in response to damaging agents.

Pso2 represents one of only a small number of proteins currently thought to function solely in repair of interstrand crosslinks (22). We tested the possibility that Pso2 might function as a 'translesional' exonuclease by removing one strand of an ICL lesion, unimpeded by the presence of a crosslink as observed for the human homologues SNM1A (34). However, when tested with a DNA substrate containing a single interstrand crosslink, Pso2 was unable to digest past the ICL lesion (Supplementary Table S1 and Supplementary Figure S5), suggesting its role in ICL repair is not associated with 'translesional' 5′-exonuclease function.

We further tested the ability of Pso2 to gain entry and degrade DNA from a nicked substrate. A short 18-bp DNA substrate (Supplementary Table S1) with an internal nick and two hairpin ends was used for simplicity. As shown in Supplementary Figure S4F, Pso2 efficiently digested the substrate from the nick site in a 5′-to 3′-direction. In addition, Pso2 appeared to generate intermediates that are consistent with opening of hairpin structures on each end of the substrate. Intermediates marked by red and blue arrows in Supplementary Figure S4F indicate species that appear to have been produced by opening hairpins closest to the 5′- and 3′-ends, respectively. Both intermediates were further degraded by Pso2 once newly generated 5′-phosphates were made available upon hairpin opening. Thus, in addition to degrading DNA from a nick, Pso2 also generated intermediates consistent with the ability to open DNA hairpins.

The rate of Pso2 nuclease activity for each of the substrates analyzed is summarized in Table 1. These findings demonstrate that the exonuclease activity of Pso2 is functional on a wide range of DNA structures as long as a free 5′-phosphate is available. Since only minor differences are observed in rates of Pso2 exonuclease activity (Table 1) it appears that Pso2 exhibits no apparent DNA structure specificity. This is in marked contrast to the human homolog of Pso2, hSNM1A, which only degrades ssDNA from its 5′-terminus.

Pso2 is a structure-specific endonuclease

Results obtained during the analysis of Pso2 exonuclease activity (Supplementary Figure S4F) suggested that Pso2 might be able to open DNA hairpin structures. To verify this activity, we analyzed the ability of Pso2 to process a doubly hairpinned substrate containing no nicks. The integrity of the fully closed hairpin structure was confirmed by treatment with both ssDNA and dsDNA exonucleases (RecJf and T7) (Figure 2B). As expected, the hairpin substrate was completely resistant to both exonuclease activities. In Figure 2B, the ability of Pso2 to open a DNA hairpin structure even when there were no nicks present in the substrate is demonstrated. A Pso2 mutant

lacking exonuclease activity (H611A) failed to open hairpin structures (Figure 1C and D) indicating that Pso2 makes use of a single active site for both endo- and exonuclease functions. Taken together with the finding that endonuclease activity does not occur on linear ssDNA or dsDNA substrates (Figure 1, Supplementary Figure S4D and E), this data strongly suggests that Pso2 is a DNA hairpin, structure-specific endonuclease.

Table 1. Pso2 nuclease activity with different DNA substrates

DNA	k_{cat} (s^{-1})
ss	0.018 ± 0.004
ds (blunt)	0.015 ± 0.0002
ds (3-overhang)	0.008 ± 0.006
ds (5-overhang)	0.005 ± 0.004
Hairpin	0.04 ± 0.01

Additionally, it also demonstrates that Pso2 is capable of opening fully paired DNA hairpins in substrates lacking a nick. This is in direct contrast to Mre11, an enzyme well characterized for its hairpin processing ability, which has severely reduced endonuclease activity towards fully paired hairpins and appears to function with Sae2 on DNA hairpins containing a single strand nick (8).

To determine the exact location of DNA strand cleavage we performed kinetic analysis of Pso2 endonuclease activity using a 3′-^{32}P-labelled hairpin substrate lacking a free 5′-PO_4. The fidelity of this substrate was established via treatment with T7 and RecJf exonuclease activities. As expected, T7 exonuclease degraded the substrate to half its original size while RecJf had no effect (Figure 2A). This substrate is only susceptible to Pso2 exonuclease activity once the hairpin has been opened. Analysis of nuclease activity with this substrate (Figure 3) demonstrated the ability of Pso2 to open

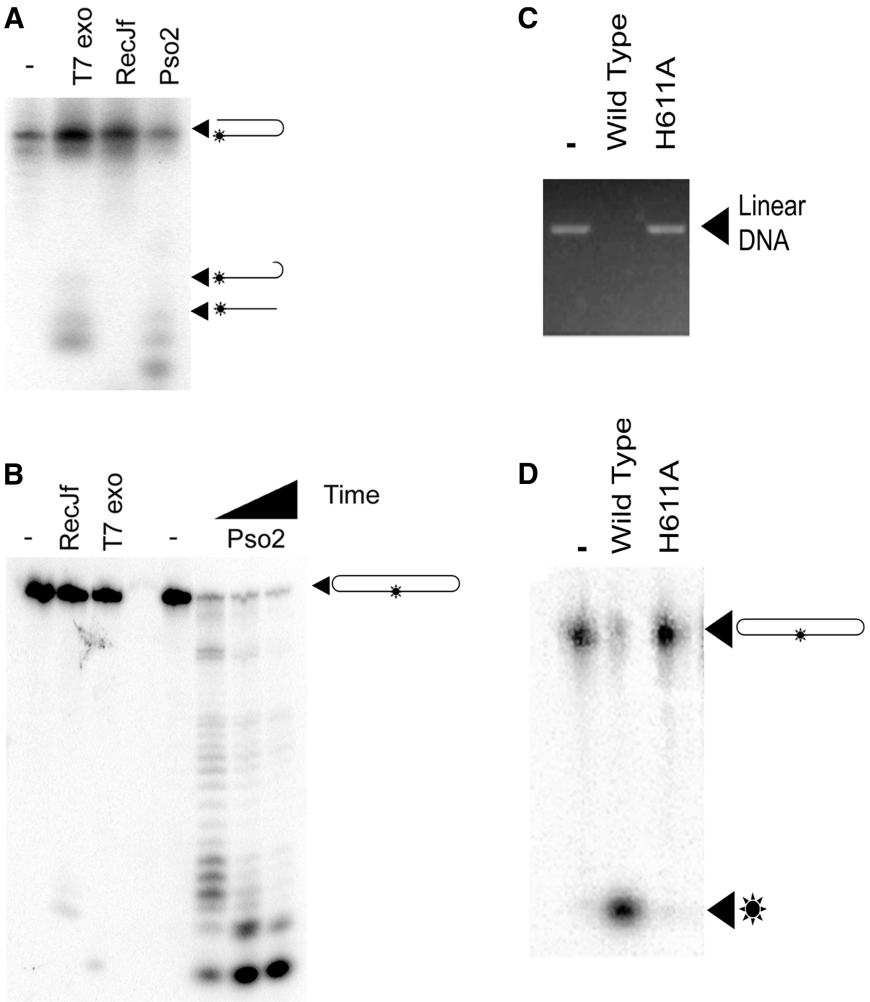

Figure 2. Pso2 opens DNA hairpin structures. DNA substrates containing either a single 3-nt hairpin (**A**) or covalently closed double 3-nt hairpin (**B**) were interrogated with T7 ds- and RecJf ss-exonuclease activities to verify structural integrity of each substrate. In (A) T7 ds-exonuclease is able to degrade the hairpin to half its original size, while RecJf had no effect since there is no ssDNA present in this substrate. Pso2 opens the hairpin on the 3′-side of the apex. In (B) neither RecJf or T7 exonuclease activities were able to degrade the fully closed double hairpin structure. Pso2 opened the hairpin structures and subsequently degraded the substrate using its 5′-exonuclease activity. (**C**) Pso2 WT and *pso2* H611A (5 μM) were assayed with linear pUC19 plasmid. *pso2* H611A shows no exonuclease activity. (**D**) Pso2 WT and *pso2* H611A (1 μM) were incubated with a completely closed hairpin substrate comprised of 20-bp duplex, two 3-nt DNA hairpins at each end and an internal label.

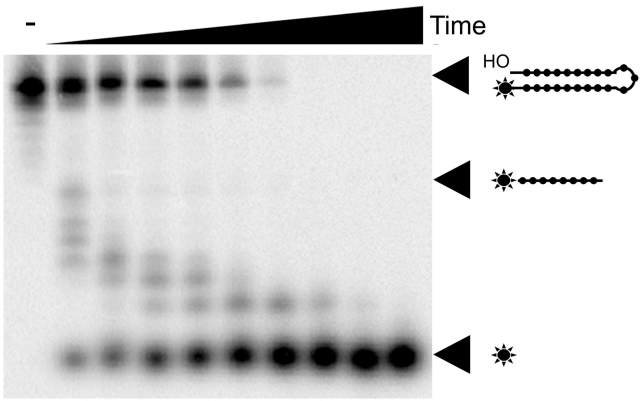

Figure 3. Kinetic analysis of Pso2 endonuclease activity. Kinetic analysis of Pso2 (80 nM) endonuclease activity was performed with a 3′-labelled DNA hairpin containing 9 bp of dsDNA, a single 3-nt hairpin and 5′-OH (100 nM). An 8-nt product is generated immediately following initiation of the reaction that is consistent with hairpin opening 2 nt from the apex on the 3′-side.

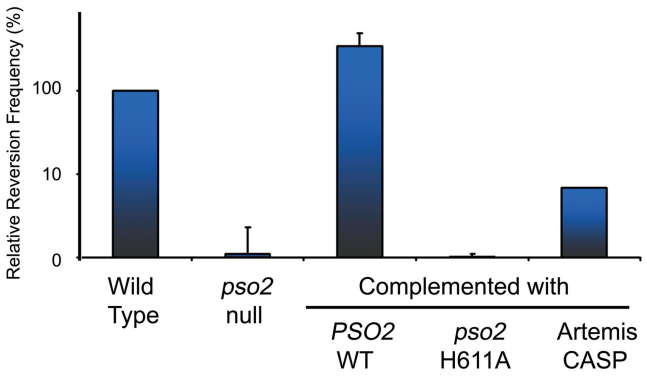

Figure 4. *In vivo* analysis of Pso2 hairpin opening activity. Reversion frequency, in percent, is plotted as a function of Pso2 status in *S. cerevisiae*. Percentages were normalized to wild-type rates of reversion. Reversion frequency correlates with repair of chromosomal DNA hairpins generated via excision of the Ac transposon from within the *ADE* gene. *Pso2* null strains have ~100-fold decreased repair, which is only restored by complementation with a wild-type *PSO2* or the β-CASP domain of Artemis.

hairpin structures on the 3′-side of the apex and subsequently remove single nucleotides from the exposed 5′-PO_4. The preferred site for cleavage was located two nucleotides away from the hairpin apex.

Pso2 displays DNA hairpin opening activity in *S. cerevisiae*

Identification of a robust Pso2 hairpin opening activity *in vitro* suggested that hairpin intermediates may be encountered and processed by Pso2 during DNA repair *in vivo*. To further investigate this possibility, we analyzed the ability of Pso2 to open chromosomal hairpin structures in an *in vivo* transposon based assay.

In this assay, an *ADE2* gene is disrupted by insertion of an Ac/Ds transposon rendering cells incapable of growth in the absence of adenine. Ac/Ds is a member of the hAT superfamily of transposons which generate hairpin structures in the flanking chromosomal sequence during transposon excision (35,36). Thus, upon induction of transposase, Ac/Ds is excised leaving DNA hairpins structures in the flanking sequence. Removal of these hairpins is required for both cell survival and reversion to an ADE+ phenotype (36). Hairpin repair was monitored by growth on drop out base media following induction of transposon excision. As expected, reversion was reduced when *pso2* was deleted. Complementation with *Pso2* restored the rate of ADE^+ revertants to wildtype levels (~100-fold) suggesting that Pso2 nuclease function was responsible for repair of hairpin structures in >95% of recovered revertants (Figure 4). The remaining revertants, recovered in the absence of Pso2 (Figure 4, bar 2), are presumably processed by the well characterized hairpin opening activities of MRX and Sae2 complexes (8,9,12,37,38). Interestingly, when Mre11 and Sae2 were deleted we were unable to recover revertants, suggesting these proteins play an important role in the repair process (Supplementary Figure S6). However, since Mre11 and Sae2 are important for end joining (6,7,39) it is difficult to assess their direct contributions in hairpin removal, as the ability to recover revertants in this assay is absolutely dependent on functional end joining.

Our data suggests that Pso2 is responsible for opening hairpin structures generated during transposon excision. If this is true, then the repair defect in a Pso2 deletion should be suppressed by an activity that is able to open hairpins. We tested this possibility by complementing a *pso2Δ* strain with the β-CASP domain of human Artemis. This domain of Artemis is constitutively active and able to open DNA hairpin structures *in vitro* and *in vivo* (40). As shown in Figure 4, the β-CASP domain of Artemis was able to suppress the repair defect in Pso2 deficient cells, suggesting that Pso2 is also able to open DNA hairpins *in vivo*.

DISCUSSION

Pso2 belongs to a small group of proteins required for repair of ICL induced damage [reviewed in (21,41)]. Of the 10 *PSO* genes characterized in *S. cerevisiae*, only *PSO2* is specifically required for repair of ICL lesions (22,41). It has been shown that Pso2 and SNM1A is essential for processing of DSBs generated during repair of ICL damage (26–28), but not breaks generated by other means including exposure to bleomycin, HO endonuclease and ionizing radiation (23,26). An obvious question then arises as to what DNA intermediate is generated during ICL repair that is unique from DSBs generated by other damaging events and that cannot be processed by nucleases functioning in other repair pathways. Despite being discovered more than 30 years ago, there are still no good mechanistic explanations for the specialized role of Pso2 in ICL repair. Our work demonstrates that Pso2 displays 5′-exonuclease activity that is independent of DNA structure, but absolutely dependant on the presence of a 5′-phosphate. Surprisingly, Pso2 also maintains a highly active hairpin-specific endonuclease activity able to open fully paired hairpin structures in *S. cerevisiae*. At what point such hairpins would occur in budding yeast is not

clear; however, our work suggests that when formed, Pso2 contributes to their resolution and repair. Together, these results identify several novel aspects of Pso2 activity, and raise questions regarding the mechanism of Pso2 and SNM1A in repair of DNA intermediates generated either directly or indirectly through exposure to ICL inducing agents.

Role of Pso2 in DNA hairpin opening

Cells utilize at least two mechanisms for hairpin processing. The first involves hairpin 'removal' and makes use of a nick close to the hairpin end (9); while the second involves 'opening' of the hairpin close to its apex and is independent of a nick (3,11) (Supplementary Figure S1). In mammalian cells, Artemis is essential for opening of fully paired hairpin structures (11). Other hairpin opening endonuclease functions, such as the MRX (MRN in mammals) complex do not efficiently open fully paired hairpins, but rather appear to mediate removal of hairpin structures generated at inverted repeat sequences typically containing larger hairpin loops (3,42). In the work reported here, we show that both Pso2 and Artemis cleave DNA hairpin structures two nucleotides from the hairpin apex on the 3′-side underscoring the similar manner in which they engage and process their substrates. We further show that a constitutively active domain of Artemis is able to partially complement a *pso2* deficient strain for repair of DNA hairpins. Given the recent findings that Artemis does not possess 5′-exonuclease activity we can conclude that it was not exonuclease function that restored the repair defect (43). Thus, our findings provide strong evidence that Pso2 acts on similar substrates processed by Artemis, further suggesting that these proteins may be more functionally related than previously appreciated. It is important to note that human SNM1A does not appear to have hairpin opening activity and has recently been shown to possess translesional exonuclease activity (33,34). Both of these functions are in contrast to the findings reported here with Pso2. It is possible that apparent differences in function are due to the particular design of experiments. This seems particularly likely, given that prior work has demonstrated that SNM1A, but not full length Apollo or Artemis, can rescue pso2 mutants (44).

Pso2 endonuclease activity in ICL repair

The mechanism of ICL repair in eukaryotes is still poorly understood. Although studies have identified key proteins in ICL repair, it is not yet clear how these proteins function to generate DNA intermediates suitable for repair. Pso2 represents one of only a small number of proteins that function predominantly in ICL repair (22). The finding that Pso2 is able to open DNA hairpin structures *in vitro* and *in vivo* suggests that this activity may be important for processing related DNA intermediates generated directly or indirectly in response to ICL damage.

How might DNA hairpins be generated during ICL damage and/or repair? Several possibilities exist. Introduction of a DNA interstrand crosslink creates a physical barrier to DNA replication, causing stalling, fork collapse and formation of DSBs (45). In support of this idea, DSBs are found to accumulate to a greater extent in actively versus non-actively replicating cells following exposure to ICL-inducing agents (46). Brendel *et al.* (22) proposed a model whereby movement of the replication fork toward an ICL lesion would cause cruciform extrusion (Figure 5). These structures could be generated on both sides of an ICL lesion due to topoisomerase I activity functioning ahead of the replication fork. Similar to cruciforms formed at long inverted repeats in yeast (3), cruciforms generated by replication toward an ICL would be converted into various sized hairpin-capped double stranded breaks (Figure 5) (10). These substrates may in turn serve as intermediates for MRX and/or Pso2 endonuclease activities. Consistent with this hypothesis, deletion of *PSO2* has been shown to result in accumulation of DSBs (26). As such, it is thought that Pso2 functions subsequent to DSB formation in ICL repair. The biochemical and genetic analysis of Pso2 presented here, is consistent with at least a subset of these DSBs being capped by hairpin structures.

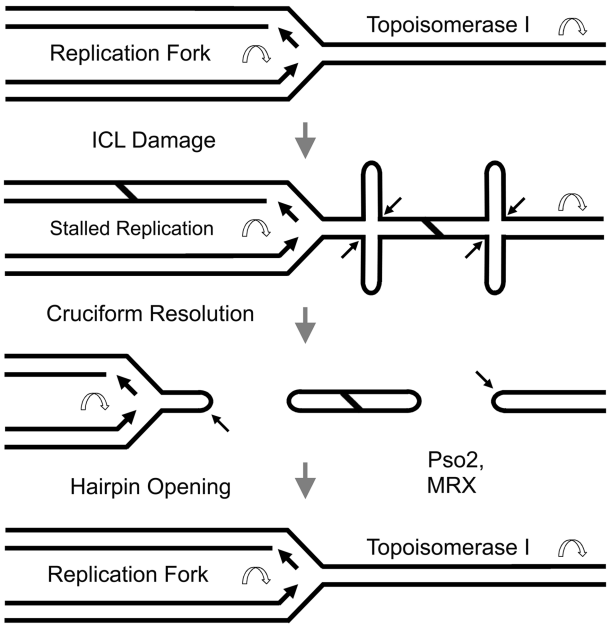

Figure 5. Model for generation and repair of hairpins during ICL damage. As the replication fork moves toward an ICL lesion helicase causes cruciform extrusion between the fork and ICL. Topoisomerase I activity ahead of the ICL also may create cruciform structures. Cruciform structures are resolved into hairpin capped DSBs (3, Cote and Lewis, 2008). Pso2 and/or the MRX/Sae2 complex process hairpin structures for subsequent end-joining repair.

A second possibility for how Pso2 might utilize its structure-specific endonuclease function in ICL would be repair not of DNA hairpins but rather of 'hairpin-like' structures. Even if a DSB were not generated close to an ICL, the distortion created by ICL damage may be sufficiently similar to a DNA hairpin for Pso2 to recognize and cleave the lesion.

In this work we have shown that Pso2 is required for processing DNA hairpins not readily accessed by other

nucleases. This phenomenon is apparent *in vivo* and is independent of ICL damage, suggesting that Pso2 may play a cellular role in DNA hairpin processing that is in addition to its specialized function in ICL repair. Furthermore, Pso2 was shown to account for >95% of the fully paired hairpin opening activity in *S. cerevisiae*, explaining its non-epistatic relationship to MRX (28). Although both these functions process DNA hairpins, they do not appear to work equally well on precisely the same intermediate. While Pso2 efficiently opens fully paired hairpin structures its activity on hairpin structures generated at long inverted repeats is apparently greatly reduced compared to the MRX complex (3).

The finding here that Pso2 has structure-specific endonuclease activity toward DNA hairpins opens up many fascinating possibilities for potential roles of Pso2 in the ability of yeast and human cells to protect themselves against DNA damage.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online: Supplementary table S1, Supplementary figures S1–S6 and Supplementary references [1–2,8–9,11,47].

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