Further characterization of the role of Pso2 in the repair of DNA interstrand cross-link-associated double-strand breaks in *Saccharomyces cerevisiae*

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DNA interstrand cross-links (ICL) are thought to be one of the most lethal forms of DNA damage. Therefore, they present a colossal challenge for the DNA damage response and repair pathways. In *Saccharomyces cerevisiae*, ICL repair utilizes factors from all of the three major repair groups: nucleotide excision repair (*RAD3* epistasis group), post-replication repair (*RAD6* epistasis group) and recombinational repair (*RAD52* epistasis group). Moreover, there are additional factors significantly influencing the repair of ICL in this organism. These have been designated *PSO1-10* based on the *pso*ralen sensitive phenotype of the corresponding mutants. Phenotype of the *pso2* mutant suggests that Pso2 is not involved in incision step of ICL repair, but it rather functions in some downstream event such as processing of DNA ends created during generation of ICL-associated double-strand breaks (DSB). In order to address the question whether function of Pso2 in the repair of ICL-associated DSB could be mediated through protein-protein interactions, we have conducted a comprehensive two-hybrid screen examining a possibility of interaction of Pso2 with Yku70, Yku80, Nej1, Lif1, Dnl4, Rad50, Mre11, Xrs2, Rad51, Rad52, Rad54, Rad55, Rad57, Rad59 and Rdh54. Here we show that Pso2 associates with none of the above DSB repair proteins, suggesting that this protein very likely does not act in the repair of ICL-associated DSB *via* crosstalk with DSB repair machinery. Instead, its function in this process seems to be rather individual.

Key words: PSO2, homologous recombination, non-homologous end-joining, DNA double-strand break repair, DNA interstrand cross-link repair

Drugs producing interstrand cross-links (ICL) between the complementary strands of the DNA represent a key component of many cancer chemotherapy regimens [1]. Although some of them also produce other types of DNA damage, there is compelling evidence that ICL are the critical cytotoxic lesion [2]. Therefore, ICL present a formidable challenge to the DNA damage response and repair pathways. As ICL represent very complex type of DNA lesion, there is no single DNA repair pathway capable of eliminating ICL. The first model for ICL repair in *Escherichia coli* [3] suggested that the sequential action of nucleotide excision repair (NER) and homologous recombination repair (HRR) pathways may be sufficient to repair ICL in an error-free manner (for a review, see [4]). In contrast to *E. coli*, ICL repair in eukaryotes has proved to be more difficult to define and the budding yeast *Saccharomyces cerevisiae* represents a powerful tool for dissecting ICL repair in lower eukaryotes. In this organism, ICL repair utilizes factors from all of the three major repair groups, NER, post-replication repair (PRR) and HRR, originally defined by the epistatic relationships of mutants in response to ultraviolet- and ionizing radiation (IR)-induced DNA damage [5], as well as genes that have been identified by subsequent studies (base excision repair factors, mismatch repair factors, *etc*.) (recently reviewed by [6]).

Genetic screens aimed at uncovering factors specifically influencing ICL repair in yeast have revealed ten distinct, *PSO1-10* ("*psoralen sensitive*"), genes to date. Whereas products of eight of them seem to be involved in the repair or tolerance of DNA damage, two Pso proteins, Pso7 and Pso8, influence processes unrelated to nucleic acid metabolism (reviewed in [6,7]). The *pso2* mutant was isolated and characterized over two decades ago [8]. Subsequent genetic studies revealed allelism between *pso2* and *snm1* [9], where the latter mutation was isolated on the basis of conferring

a specific sensitivity to nitrogen mustard [10, 11]. Although genetic studies initially assigned *PSO2* to the *RAD3*/NER epistasis group for sensitivity to cross-linking agents, there are fundamental distinguishing features between the cells inactivated in the *PSO2* and NER genes with respect to ICL repair. Whereas *pso2* cells produce both DNA single- and double-strand breaks (SSB and DSB, respectively) after 8-methoxypsoralen photoaddition though are unable subsequently to reconstitute double-stranded DNA, the *rad3* mutants are impaired in the ICL incision step [12]. Hence *pso2* mutants are proficient in the incision step of ICL repair, but are defective at some downstream processing event. Further investigations into the genetic relationships of *PSO2* with other repair pathways showed a lack of epistasis with the *RAD52*/HRR epistasis group [13]. Data regarding the interaction with the *RAD6*/PRR pathway are rather inconsistent.

The Pso2 protein is a member of the metallo-β-lactamase superfamily of enzymes that share a conserved hydrolytic domain possessing metal-binding sites [14, 15]. Pso2 possesses 5'-nuclease activity on single- and double-stranded oligonucleotide substrates [16], and this activity is dependent upon an active metallo-β-lactamase domain [16, 17]. The Pso2 protein also possesses a second domain highly conserved in metallo-β-lactamase-containing family of nucleic acid processing enzymes, namely a β-CASP domain, self-identified by the key members of this family (<u>C</u>PSF, <u>A</u>RTEMIS and <u>S</u>nm1/<u>P</u>so2) [15]. CPSF is an mRNA processing enzyme [18], whereas ARTEMIS (when complexed with DNA-PKcs) is an endonuclease that plays a relatively well-defined role in V(D)J recombination [19], as well as likely playing a nucleolytic role in non-homologous end-joining (NHEJ) [20, 21].

In *S. cerevisiae* cells treated with ICL-forming agents, DSB are generated during repair of ICL. It appears that DSB generation is highly dependent upon ICL induction and reaches its maximum closely after the ICL treatment [12, 22]. It is believed that DSB are not obligate intermediates of ICL repair but result when the replication forks are stalled at the unprocessed or partially repaired ICL, an assumption that is substantiated by a number of findings [22–24]. ICL-associated DSB apparently differ from those created by IR or restriction endonucleases, as they are much more slowly repaired [25]. In the wild type cells, the majority of ICL-associated DSB can be rejoined within 24 hours [12], whereas this process is significantly impaired in the *pso2* mutant cells. However, IR resistance and repair of HO endonuclease-induced DSB are normal in the absence of Pso2, suggesting that the protein is likely not involved in general DSB repair, but controls a step specific in ICL repair [8, 17]. Both DSB repair pathways, HRR (reviewed in [26-28]) and NHEJ (reviewed in [28–30]), operate on ICL-associated DSB, although NHEJ may function only as a minor, back-up, system that probably acts on ICL-induced DSB if HRR is impaired.

Given the fact that ICL are associated with DSB in *S. cerevisiae*, a relevant question is whether Pso2 physically interacts with DSB repair factors. To answer this question, we conducted a comprehensive two-hybrid screen using Pso2 and all known DSB repair proteins expressed from bait and prey plasmids, respectively. Our data show that Pso2 associates with none of the DSB repair proteins, suggesting that Pso2 likely does not act in ICL repair *via* crosstalk with DSB repair machinery and that this protein likely acts in DNA-ends processing step during the repair of ICL-associated DSB as an individual factor.

Table 1. Primers used in this study

Gene amplified	Primer	Sequence (5' to 3')
PSO2	PSO2(NcoI)for PSO2(BamHI)rev	CG*CCATGG*TT**ATG**TCAAGGAAATCTATAGTGCAAA[a, b] GC*GGATCC*TTT**TTA**TTTAGCCGCCCGCGTTTTCCTA[b, c]
RAD52	RAD52(NcoI)for RAD52 (EcoRI)rev	CG*CCATGG*TGTTG**ATG**AATGAAATTATGGATATGGATGA CG*GAATTC*GTCGACT**TCA**AGTAGGCTTGCGTGCATGCAG
RAD54	RAD54(NcoI)for RAD54(EcoRI)for	CATC*CCATGG*CG**ATG**GCAAGACGCAGATTACCAGAC CG*GAATTC***TCA**ATGTGAAATATATTGAAATGC
RDH54	RDH54(NcoI)for RDH54(BamHI)rev	CATC*CCATGG*CG**ATG**CAGATACCGAAATATGAGAAC CG*GGATCC***TCA**TTGTTCTCTGAGACATATCTC
RAD55	RAD55(NcoI)for RAD55(BamHI)rev	CATC*CCATGG*CG**ATG**TCGCTTGGTATACCACTTTCCC CG*GGATCC***TTA**ACCTTCACTATCATAAATTATC
RAD57	RAD57(NcoI)for RAD57(BamHI)rev	CATC*CCATGG*CG**ATG**CCTAGGGCCTTATCAATAAAA CG*GGATCC***TCA**GGCTGTTTCTATTCCTCGCTTAGT
RAD59	RAD59(NcoI)for RAD59(BamHI)rev	CATC*CCATGG*CG**ATG**ACGATACAAGCGAAGCCCAGT CG*GGATCC***TTA**TTTGATATGCGTGCCTTTAGC

[a] START codon is shown in bold

[b] restriction enzyme recognition sites are shown in italics

[c] STOP codon is shown in bold

Material and methods

Media and plasmids. Yeast and bacterial media, as well as all the standard yeast genetic methods, were used as described previously [31]. For Gal4-based two-hybrid screen, pAS2-1 (designed to produce fusions to the Gal4 DNA-binding domain – Gal4 BD), pGADT7 (designed to produce fusions to the Gal4 transcriptional activation domain – Gal4 AD) and pCL1 (encodes full-length, wild type Gal4, and therefore provides a positive control for β-galactosidase assay) vectors from CLONTECH Laboratories, Inc. (Mountain View, CA, USA) were used. *RAD54, RDH54, RAD55,* RAD57 and RAD59 coding sequences were amplified from genomic DNA by PCR using the primers listed in Table 1. The PCR products were digested with appropriate restriction enzymes and cloned in frame into pGADT7 to generate recombinant vectors pGADT7-RAD54, pGADT7-RDH54, pGADT7-RAD55, pGADT7-RAD57 and pGADT7-RAD59, respectively. The *RAD51* and *RAD52* coding sequences were sub-cloned in frame from pGBT9-RAD51 (kindly provided by L. Krejčí, Masaryk University Brno, Czech Republic) [32] and pACTII-RAD52 vectors (our laboratory stock) into pGADT7 to produce pGADT7-RAD51 and pGADT7-RAD52, respectively. To generate pACTII-RAD52, *RAD52* specific PCR primers listed in Table 1 were used. The *RAD52* PCR product was digested with appropriate restriction enzymes and cloned in frame into pACTII. The *YKU70, YKU80, DNL4, LIF1, MRE11, XRS2* and *RAD50* genes were sub-cloned in frame from pACTII-YKU70, pACTII-YKU80, pACTII-DNL4, pACTII-LIF1, pACTII-MRE11, pACTII-XRS2 and pACTII-RAD50 vectors (kindly provided by S. U. Åström, Stockholm University, Sweden) [33], respectively, into pGADT7 to generate the respective pGADT7 recombinant derivatives. *NEJ1* coding sequence was sub-cloned in frame from pAS2-1-NEJ1 (kindly provided by S. U. Åström, Stockholm University, Sweden) [33] into pGADT7 to yield pGADT7-NEJ1. *PSO2* coding sequence was amplified from genomic DNA using the primers from Table 1. After digestion with suitable restriction enzymes, the *PSO2* PCR product was cloned in frame into pAS2-1 to yield pAS2-1-PSO2. pGEH009 (contains entire *DNL4* ORF cloned into pAS2-1) [34] that in combination with pGADT7-LIF1 served as a positive control in the two-hybrid screen was kindly provided by P. Schär (University of Basel, Switzerland). After being cloned into the respective two-hybrid vector, the *RAD52, RAD54, RDH54, RAD55, RAD57, RAD59* and *PSO2* PCR products were verified by DNA sequencing using an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). All two-hybrid constructs generated by sub-cloning from provided plasmids were sequenced across the fusion junction to ensure that the junctions were in frame.

Two-hybrid analysis. Pairwise combinations of the two-hybrid vectors were transformed into both Y187 (*MAT*α, *ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met⁻, gal80Δ, URA3::GAL1*ᵤₐₛ*-GAL1*ₜₐₜₐ*-lacZ*) (CLONTECH Laboratories, Inc.) [35] and PJ69-4A (*MAT*a, *ura3-52, his3-200, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, LYS2::GAL1-HIS3, GAL2-ADE, met2::GAL7-lacZ*) (kindly provided by P. James, University of Wisconsin, USA) [36] strains. Colonies from these transformations were picked after growth on selective plates lacking tryptophane and leucine for 3-5 days. Individual colonies from Y187 transformation were grown in liquid cultures under selection for both vectors, and extracts from these cells were prepared and assayed for β-galactosidase reporter gene expression level according to CLONTECH Yeast Protocols Handbook: o-nitrophenyl β-D-galactopyranoside (ONPG; Sigma-Aldrich) was used as substrate. β-galactosidase units were calculated as described previously [37]. Individual colonies from PJ69-4A transformation were also grown in liquid cultures under selection for both vectors, but the cultures were then spotted onto (i) complete plate, (ii) selective plate lacking adenine to monitor *ADE2* reporter gene expression level, (iii) selective plate lacking histidine and containing 2 mM 3-amino-1,2,4-triazole (3-AT; Sigma-Aldrich) to monitor *HIS3* reporter gene expression level, and (iv) selective plate lacking both adenine and histidine and containing 2 mM 3-AT to monitor both reporter gene expression levels at the same time. In both liquid culture and plate two-hybrid assays, Dnl4 + Lif1 pairwise combination was used as a positive control.

Results and Discussion

Currently very little is known about repair of ICL-associated DSB. It is thought that very early step in this process in yeast is under the control of the Pso2 protein. Such step very likely does not exist in general DSB repair, as *pso2* cells are not IR sensitive and do not suffer from defect in the repair of HO endonuclease-induced DSB [8, 17]. This suggests that very specific DNA-end structures are possibly created during generation of ICL-associated DSB, and that such structures are substrates for Pso2, as *pso2* cells can generate DSB after ICL treatment, but are not able to subsequently repair them [12]. This assumption is in line with recent results showing that Pso2 possesses 5'-nuclease activity [16].

In the light of above findings, we wished to determine whether there is physical interaction of Pso2 with DSB repair machinery. To achieve this, Gal4-based two-hybrid system was used [38]. In this system, interaction between two proteins, which are respectively fused to the Gal4 BD and AD, leads to activation of the reporter gene which is under the control of the *GAL* promoter region. Accordingly, the sequence encoding Gal4 BD was fused in frame with Pso2 in pAS2-1 two-hybrid vector. Similarly, all known *S. cerevisiae* DSB repair proteins [26,27,29,30], namely Yku70, Yku80, Nej1, Lif1, Dnl4, Rad50, Mre11, Xrs2, Rad51, Rad52, Rad54, Rad55, Rad57, Rad59 and Rdh54, were fused in frame to sequence encoding Gal4 AD in pGADT7 two-hybrid vector. To detect possible interaction of Pso2 with DSB repair proteins, pairwise combinations of these fusion protein expression vec-
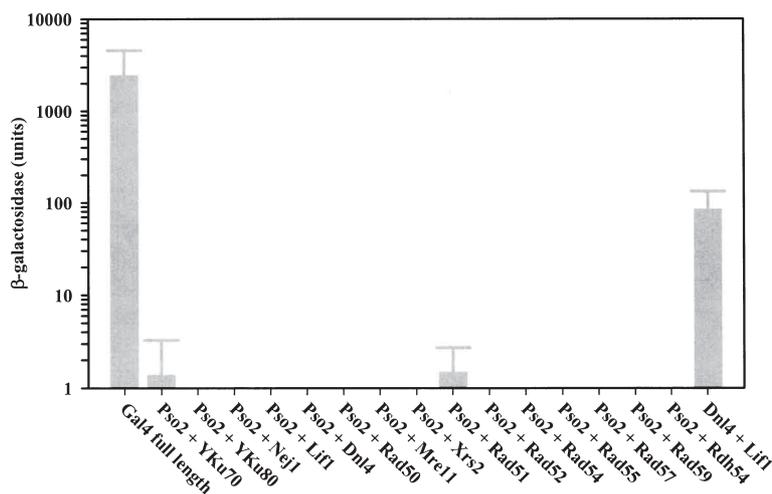
Figure 1. Results of two-hybrid analysis to detect possible interaction of Pso2 with Yku70, Yku80, Nej1, Lif1, Dnl4, Rad50, Mre11, Xrs2, Rad51, Rad52, Rad54, Rad55, Rad57, Rad59, and Rdh54. Liquid culture β-galactosidase assay and ONPG as substrate were used to quantify the possibility of above interactions. The error bars represent the standard deviations for at least three repetitions.
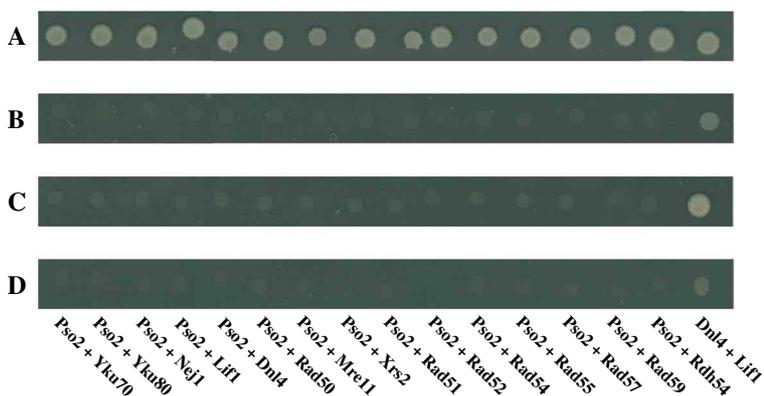

Figure 2. Results of two-hybrid analysis to detect possible interaction of Pso2 with Yku70, Yku80, Nej1, Lif1, Dnl4, Rad50, Mre11, Xrs2, Rad51, Rad52, Rad54, Rad55, Rad57, Rad59, and Rdh54. Plate assay was used to qualitatively examine the possibility of above interactions: (A) complete plate, (B) selective plate lacking adenine to monitor *ADE2* reporter gene expression, (C) selective plate lacking histidine and containing 2 mM 3-AT to monitor *HIS3* reporter gene expression, and (D) selective plate lacking both adenine and histidine and containing 2 mM 3-AT to monitor both reporter gene expressions at the same time.

β-galactosidase units, respectively) (Figure 1), we consider these levels to be insufficient to indicate a significant interaction (see text below for β-galactosidase expression level in a positive control sample Dnl4 + Lif1). Nevertheless, they still may indicate a weak or transient interaction, a situation which could not finely be detected in plate two-hybrid assay (Figure 2). Next we tested a possibility whether DNA damage could be a prerequisite factor that mediates interaction of Pso2 with DSB repair machinery, and therefore we treated the corresponding yeast cultures with a low concentration of the ICL agent, cisplatin (CDDP), for 1 and 3 h before assaying them for β-galactosidase expression levels. However, CDDP-induced DNA damage did not change interaction pattern in any of the pairwise combinations examined (data not shown), indicating that DNA damage is not able to trigger association of Pso2 with DSB repair proteins.

In order to convince others of our negative result, we ensured that our two-hybrid system was well controlled in several respects. First, we used two reporter strains [35,36], in which expression level of up to three different reporter genes, *lacZ*, *ADE2* and *HIS3*, could be monitored. This eliminated possibility that our negative two-hybrid result was influenced by strain background or strength of the particular *GAL* promoter construct. Second, full-length, wild type Gal4 expressing vector was included in our screen, enabling us to control functionality of the β-galactosidase assay. Since high β-galactosidase expression level was obtained for full-length Gal4 (2524.5 β-galactosidase units) (Figure 1), satisfactory sensitivity of β-galactosidase assay was achieved. Third, Dnl4 + Lif1 combination was included into our system as a positive control (Figures 1 and 2), as Dnl4 and Lif1 were shown to form a very stable complex [34,39]. Expectedly, our screen confirmed interaction between these two proteins (86.9 β-galactosidase units), a result that provides very important validation of the functionality of our screen. Based on above facts, we conclude that negative result of our two-hybrid screen was indeed caused by no interaction between the proteins examined. Nevertheless, we cannot exclude a possibility that the use of different approach or truncated versions of some pairwise combinations could overcome our inability to detect significant interaction between Pso2 and DSB repair proteins.

tors (for details, see Figures 1 and 2) were transformed into two reporter strains, Y187 and PJ69-4A, and tested for their ability to activate reporter gene expressions: combinations created in Y187 were assayed for β-galactosidase reporter gene expression level (Figure 1), whereas those in PJ69-4A were assayed for *ADE2* and *HIS3* reporter gene expression levels (Figure 2).

As obvious, Pso2 associates with none of the DSB repair proteins in our two-hybrid system (Figures 1 and 2). Although there were low β-galactosidase expression levels in Pso2 + Rad51 and Pso2 + Yku70 pairwise combinations (1.5 and 1.4

In summary, we suggest that the Pso2 protein likely acts in DNA-ends processing step during repair of ICL-associated DSB as an individual factor. Whether or not it requires for this role some interacting partner(s) outside of DSB repair pathways remains to be elucidated.

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