Disruption of the RAD51 Gene Sensitizes S. cerevisiae Cells to the Toxic and Mutagenic Effects of Hydrogen Peroxide

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Received 18 November 2003

ABSTRACT. The RAD51 gene was disrupted in three different parental wild-type strains to yield three rad51 null strains with different genetic background. The rad51 mutation sensitizes yeast cells to the toxic and mutagenic effects of H2O2, suggesting that Rad51-mediated repair, similarly to that of RecA-mediated, is relevant to the repair of oxidative damage in S. cerevisiae. Moreover, pulsed-field gel electrophoresis analysis demonstrated that increased sensitivity of the rad51 mutant to H2O2 is accompanied by its decreased ability to repair double-strand breaks induced by this agent. Our results show that ScRad51 protects yeast cells from H2O2-induced DNA double-strand breakage.

Reactive oxygen species generated by normal metabolism or produced by exogenous agents can induce several types of DNA damage including base damage, base loss and strand breaks (Demple and Har- rison 1994; Wallace 1998). Oxidative DNA damage is considered to be the most significant endogenous DNA damage; approximately 2 × 10^4 oxidative DNA lesions occur per human genome every day. The repair of these lesions is assumed to have a central role in preventing living organisms from increasing mutation rates. Organisms have evolved highly efficient enzyme mechanisms that function in the repair of oxidative DNA damage. The majority of these DNA lesions, such as impaired bases and single-strand breaks (SSB), are repaired via the base excision repair (BER) pathway, while DNA double-strand breaks (DSB) are supposed to be repaired predominantly by enzymes involved in homologous recombination (HR) (Memisoglu and Samson 2000; Wallace 2002). Moreover, other repair mechanisms including nucleotide excision repair (NER) and lesion bypass pathway have been shown to have an overlapping specificity in the removal of, or tolerance to, oxidative DNA damage in S. cerevisiae (Swanson et al. 1999; Doetsch et al. 2001). Recent studies on mammals point also to the role of nonhomologous end-joining (NHEJ) in the processing of DSB generated by oxidizing agents (Kristoffer and Povirk 2003).

In E. coli, DSB repair occurs exclusively by HR and the RecA protein plays a pivotal role in this process (Kowalczykowski et al. 1994; Bianco et al. 1998). That RecA-mediated repair is relevant to the repair of oxidative DNA damage is evident from the finding that the E. coli recA mutants are very sensitive to killing by hydrogen peroxide (H2O2) (Carlsson and Carpenter 1980; Hagensee and Moses 1986; Imlay and Linn 1987; Konola et al. 2000).

The Rad51 protein (ScRad51) in S. cerevisiae is considered to be a structural and functional homolog of the RecA protein (Ogawa et al. 1993; Game 2000; Sung et al. 2000). Among the members of the RAD52 epistasis group of genes involved in DSB repair by HR in yeast, RAD51 is considered to be very important and highly conserved among eukaryotes (Sung et al. 2003). The key role of ScRad51 resides in the search for homologous DNA and DNA strand exchange reactions which are performed by remarkably similar mechanisms in bacteria and yeast (Vispe and Defais 1997; Baumann and West 1998). Expectedly, due to impaired DSB repair, rad51 mutant cells are sensitive to agents that induce DSB such as ionizing radiation (IR) (Game and Mortimer 1974; VandenBosch et al. 2002), methyl methanesulfonate (MMS) (Donovan et al. 1994; Morais et al. 1996), bleomycin (Blm) (Moore 1978; Abe et al. 1994), and photo-activated 8-methoxypsoralen (8-Mop + UVA) (Morais et al. 1996, 1998).
Because of structural and functional similarity between RecA and ScRad51, the H$_2$O$_2$ sensitivity of the rad51 mutant cells has been examined in the present study. We show that a rad51 mutation sensitizes yeast cells to the toxic and mutagenic effects of H$_2$O$_2$, suggesting that Rad51-mediated repair, similarly to the RecA-mediated one, is relevant to the repair of oxidative damage in S. cerevisiae. Moreover, using pulsed-field gel electrophoresis (PFGE) analysis we demonstrated that increased sensitivity of the rad51 mutant to H$_2$O$_2$ is accompanied by decreased ability to repair DSBs created by this agent.

**MATERIALS AND METHODS**

*Chemicals and enzymes.* Hydrogen peroxide (30 %, W/W), proteinase K, lyticase, N-laurylsarcosine and phenylmethanesulfonyl fluoride (PMSF) were from Sigma. Low-melting point agarose (inCert® agarose) and agarose for PFGE (FastLane® agarose) were purchased from FMC BioProducts (USA). Pefabloc® SC and 2-mercaptoethanol (2-ME) were from Serva (Germany).

*Yeast strains and construction of rad51 mutants.* S. cerevisiae strains used are listed in Table I. The rad51 mutants were generated by disruption of the RAD51 gene using the disruption plasmid p51::LEU2 kindly provided by Dr. F. Fabre (CEA, Fontenay-aux-Roses, France). The plasmid was digested with BamHI to isolate a 4.5-kb fragment bearing the rad51::LEU2 allele, which was introduced into three different parental wild-type strains (Table I). The positive clones were obtained by selection for Leu$^+$ phenotype. The genotype of mutants bearing the disruption of the RAD51 gene was confirmed by PCR or Southern blot analysis.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>F808</td>
<td>MATa ade1-100 his4-519 leu2-3,112 ura3-52</td>
<td>D. Keszenman, LOBBM, Montevideo, Uruguay</td>
</tr>
<tr>
<td>F3-51</td>
<td>MATa ade1-100 his4-519 leu2-3,112 ura3-52 rad51::LEU2</td>
<td>this study</td>
</tr>
<tr>
<td>FF18733</td>
<td>MATa his7-2 leu2-3,112 lys2-1 trp1-289 ura3-52</td>
<td>F. Fabre, CEA, Fontenay-aux-Roses, France</td>
</tr>
<tr>
<td>AD33-51</td>
<td>MATa his7-2 leu2-3,112 lys2-1 trp1-289 ura3-52 rad51::LEU2</td>
<td>this study</td>
</tr>
<tr>
<td>YPH252</td>
<td>MATα ura3-2 lys2-801 ade2-101 trp1-ΔI his3-Δ200 leu2-ΔI</td>
<td>J.A.P. Henriques, Centro de Biotecnologia UFRGS, Porto Alegre, Brazil</td>
</tr>
<tr>
<td>YPDV-51</td>
<td>MATα ura3-2 lys2-801 ade2-101 trp1-ΔI his3-Δ200 leu2-ΔI rad51::LEU2</td>
<td>this study</td>
</tr>
</tbody>
</table>

*Media and growth conditions* were the same as described by Brozmanová et al. (2001). Briefly, yeast cells were nonselectively grown in YPD medium (in %: bacto-peptone 2, agar 2, yeast extract 1). Synthetic complete (SC) medium (0.67 % yeast nitrogen base, 1 % diammonium sulfate and essential nutrients) lacking leucine with 2 % glucose and 2 % agar was used for selective growth of transformed cells, while medium lacking histidine was used for determination of His$^+$ revertants. SC plates with canavanine (60 µg/mL) were used for determination of mutants resistant to canavanine.

*Cellular sensitivity measurement.* H$_2$O$_2$ treatment was carried out with exponentially growing cells. Briefly, yeast cells were cultivated overnight in SC medium and then used for inoculation into YPD. Incubation in YPD continued until the cell suspension reached cell concentration 10/nL (i.e. 10$^7$/mL). The cells were resuspended in 0.1 mol/L potassium phosphate (pH 7.4) buffer; aliquots were shaken for 1 h at 30 °C with H$_2$O$_2$. After the treatment, the cells were collected by centrifugation, washed twice with 0.1 mol/L potassium phosphate and resuspended in the same buffer. Appropriate dilutions were plated on YPD to determine cell killing; SC plates lacking histidine or supplemented with canavanine were used to determine His$^+$ reversion or forward mutation frequency, respectively.

*Pulsed-field gel electrophoresis* was done according to Brozmanová et al. (2001). Briefly, the treated cells were washed twice with 50 mmol/L EDTA (pH 7.5) and resuspended in the same solution to give a cell concentration of 312.5/nL (i.e. 3.125 × 10$^8$/mL). Thereafter, 5 × 10$^7$ cells were mixed with 40 µL SCE buffer (in mol/L: glucitol 2, citrate 1, EDTA 0.5; pH 7.5) containing 10 % 2-ME and 5 µg lyticase (380 U/mg); 200 µL 1 % low-melting-point agarose in 0.125 mol/L EDTA (pH 7.5) was then added. The
resulting cell suspension was equilibrated at 42 °C, immediately transferred into the plug moulds and cooled until solidified. After removing from the moulds, the plugs were incubated in 0.5 mol/L EDTA, 0.4 % 2-ME and 10 mmol/L Tris-HCl (pH 8.0) for 2 h and lysed at 37 °C overnight in 0.5 mol/L EDTA, 10 mmol/L Tris-HCl, 1 % N-laurylsarcosine and 0.5 mg/mL protease K. Next day, the plugs were incubated at 37 °C for 2 h in a buffer containing (in mmol/L: Tris-HCl 10, EDTA 1, PMSF 1; pH 7.0) and then rinsed twice with 50 mmol/L EDTA (pH 7.5). The plugs were stored at 4 °C in (mmol/L): Tris-HCl 10, EDTA 10, PMSF 1 (pH 7.5) until use.

Before loading into the wells, the plugs were equilibrated twice in 10 mmol/L Tris-HCl and 1 mmol/L EDTA (pH 8.0). Loaded wells were covered with a 1 % agarose gel. Electrophoresis was done with 1 % agarose gel and TAE buffer (20 mmol/L Tris–acetate, 1 mmol/L EDTA; pH 8.0) using a transverse alternating field electrophoresis system (Beckman Instruments) at 14 °C: (a) constant current of 170 V/mA for 30 min with 4 s pulse time and (b) constant current of 150 V/mA for 20 h with 90 s pulse time. After electrophoresis, the gels were stained with 0.1 µg/mL ethidium bromide, destained in TAE buffer, visualized on a UV transilluminator and photographed with GDS 7500 Gel Documentation System (UVP).

RESULTS AND DISCUSSION

Cell survival. To confirm that ScRad51 plays a role in the H2O2 resistance in yeast, the coding region of the RAD51 gene in three different wild-type strains was disrupted; the resulting mutant strains, bearing the disruption allele rad51::LEU2, were tested for cell survival after exposure to H2O2. All rad51 mutants showed increased sensitivity to H2O2 relative to the isogenic parental strain (Fig. 1). The differences (approximately one order of magnitude at doses higher than 100 µg/mL) were observed in all rad51 strains. These data indicate that ScRad51 is required to attenuate the toxicity of H2O2 in yeast cells.

Mutation frequency. The mutation frequency was estimated by measuring the level of either His+ reversions in the rad51 mutants derived from F808 and FF18733, or forward mutations in the CAN1 locus for rad51 mutant derived from YPH252. All rad51 strains showed moderate increase in spontaneous as well as H2O2-induced mutation frequency compared to the isogenic wild types (Fig. 2), suggesting a role of ScRad51 in eliminating the promutagenic lesions induced by H2O2. Increased spontaneous mutation frequencies of the rad51 strains correlate with the mutator phenotype of the rad52 strain belonging to the same epistasis group of genes (Paques and Haber 1999; Doetsch et al. 2001). The increased H2O2-induced mutation frequency in the absence of ScRad51 supports the existing view that DSB repair by HR is a high-fidelity process.

DNA double-strand breakage. To investigate whether the observed biological consequence of the RAD51 gene disruption, i.e. increased sensitivity and mutation frequency after H2O2 treatment, could be the result of decreased ability to repair DSBs, we monitored H2O2-induced DNA strand breakage in both the rad51 mutant and corresponding wild-type strains using PFGE. Negligible DNA fragmentation was observed after H2O2 treatment in both wild-type strains (Fig. 3; F808 and FF18733), whereas chromosomal DNA of the corresponding rad51 mutants was more fractionated mainly at the highest doses of H2O2. Similar profiles were obtained for the rad51 strain derived from YPH252 (data not shown). Our results indicate that ScRad51 is involved in protection of yeast cells from DNA double-strand breakage caused by H2O2.

We provide the first evidence that the absence of ScRad51 sensitizes the S. cerevisiae cells to the toxic and mutagenic effects of H2O2 and slows down the repair of DNA DSB. In this respect, elimination of the ScRad51 activity in S. cerevisiae seems to have similar negative effect on H2O2 sensitivity as described for the E. coli recA mutant. Because H2O2 induces SOS response in E. coli cells, hypersensitivity of the recA mutant to H2O2 is, at least partly, the consequence of their inability to induce SOS response and to increase the level of RecA protein by H2O2 treatment. Whether similar mechanisms have also been adopted by yeast cells to protect themselves against oxidative stress is unknown. The fact that ScRad51 is transcriptionally induced by DNA-damaging agents (Basile et al. 1992; Mercier et al. 2001; Gasch et al. 2001) suggests its possible potential role in a complex DNA damage-inducible SOS-like repair response. Our results demonstrating the attenuation of repair activities of the H2O2-treated rad51 mutants support this idea.

In contrast to E. coli, where a single RecA protein participates in virtually all HR events, HR in eukaryotic organisms is mediated by a group of genes known as the RAD52 epistasis group. The RAD52 group members in the yeast S. cerevisiae include RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, RDB54/TID1, MRN11 and XRS2 (Sung et al. 2000; Symington 2002). Among them, the products of the RAD51 and RAD52 genes are supposed to be the key recombination proteins. Even though rad52 mutants show severe recombination defects and very high sensitivity to the DSB-inducing agents, such as IR,
MMS, Blm, cisplatin, N-methyl-N’-nitro-N-nitrosoguanidine, and 8-Mop + UVA (for a review see Dudáš and Chovanec 2004), they display, in contrast to the rad51 mutants, only negligible, if any, sensitivity to H₂O₂ (Swanson et al. 1999; Doetsch et al. 2001; Škorvaga et al. 2003; our unpublished results). As reported earlier, more pronounced sensitivity of yeast cells to the lethal and mutagenic effects of H₂O₂ could be observed only if simultaneous disruptions of the BER enzymes (Ntg1, Ntg2, Apn1) were generated in the rad52 background (Swanson et al. 1999; Doetsch et al. 2001). This suggests the existence of a RAD52-dependent backup system in removal of, or tolerance to, oxidative DNA damage in S. cerevisiae. Lack of sensitivity of the rad52 single mutants to H₂O₂ indicates that primary and secondary oxidative DNA lesions are promptly and efficiently repaired by repair pathways which do not require the assistance of ScRad52. As evident from our results, this is not the case for ScRad51. Disruption of the RAD51 gene makes yeast cells moderately, but significantly, more sensitive to the toxic and mutagenic effects of H₂O₂ and leads to the accumulation of unrepaired DSBs. This could suggest that ScRad51 plays a slightly different and maybe more important role than ScRad52 in eliminating of the H₂O₂-induced toxic and mutagenic lesions from DNA.

Fig. 1. Sensitivity (%) of parental (open symbols) and rad51 mutant (closed symbols) strains to H₂O₂ (µg/mL); survival of F808 and F3-51 (A), FF18733 and AD33-51 (B), YPH252 and YPDV51 (C).

Fig. 2. His⁶ reversion (A, B) and forward mutation (C) frequencies (n × 10⁻⁷) of parental (F808, FF18733, YPH252; circles) and rad51 mutant (F3-51, AD33-51, YPDV51; squares) strains after treatment with H₂O₂ (µg/mL); reversions of F808 and F3-51 (rad51) (A), reversions of FF18733 and AD33-51 (rad51) (B), and forward mutations of YPH252 and YPDV51 (rad51) (C).
ScRad52 participates in at least three DSB repair pathways. Two of them, single-strand annealing (SSA) and break-induced replication (BIR), are RAD51-independent (Paques and Haber 1999; Sung et al. 2000). In the absence of ScRad51, however, S. cerevisiae cells are not able to repair DSBs by gene conversion and reciprocal exchange – the two conservative HR events that require also ScRad52. This leads to speculation that HR by gene conversion, or reciprocal exchange, are the mechanisms which are preferentially used in processing of DSB induced by oxidation in yeast. ScRad51 could have, however, some specific role in this process that does not require ScRad52 activity. This is partly supported by the recent finding (Lundin et al. 2003) showing that in mammalian cells the Rad51 protein is involved in at least two different HR pathways repairing a wide range of DNA lesions during DNA replication.

This work was supported by grants 2/3091/23 and 1/0043/03 from VEGA Grant Agency of the Slovak Republic.

REFERENCES


