Inactivation of YAP1 Enhances Sensitivity of the Yeast RNR3-lacZ Genotoxicity Testing System to a Broad Range of DNA-Damaging Agents

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Despite the great advances by using microorganism-based genotoxicity testing systems to assess environmental genotoxic compounds, most of them respond poorly, particularly to oxidative agents. In this study, we systematically examined the RNR3-lacZ reporter gene expression in Saccharomyces cerevisiae mutant strains defective in the protection against reactive oxygen species and found that only YAP1 deletion resulted in a significant enhancement in the detection of oxidative damage. To our surprise, YAP1 deletion also caused an increased cellular sensitivity to a variety of DNA damage. This altered sensitivity appears to be independent of oxidative damage because under conditions in which vitamin C treatment rescued oxidative damage, it failed to reverse the phenotypes caused by other types of DNA damage. Furthermore, although inactivation of cell permeability genes enhanced the RNR3-lacZ detection sensitivity particularly to large molecular weight compounds, their effects on small molecular oxidative agents are minimal. Taken together, this study helps to create a hypersensitive genotoxicity testing system to a broad range of DNA-damaging agents by deleting a single yeast gene.

Key Words: RNR3-lacZ; yeast; oxidative compounds; YAP1; genotoxicity test; sensitivity.

Reactive oxygen species (ROS), such as the superoxide radical, hydroxyl radical, and hydrogen peroxide (H2O2), generated by endogenous and exogenous sources, cause significant damage to macromolecules, including DNA, lipids, proteins, and other macromolecules (Apel and Hirt, 2004). DNA damage caused by ROS includes a large variety of lesions: e.g., base and sugar damage, rearrangements, deletions, insertions, and DNA-protein cross-links (Friedberg et al., 2006). Oxidative DNA damage may play a central role in both aging and various age-related degenerative diseases, including cancer (Ames, 1983; Bal and Kasprzak, 2002). Because of the widespread biological effects of ROS, organisms either grow anaerobically or have evolved ways to avoid and repair oxidative damage to DNA (Apel and Hirt, 2004; Frei et al., 1988). Like other organisms, yeasts possess many defense systems against oxidative damage, including chemical antioxidants, DNA repair enzymes, catalase, and superoxide dismutase (Jamieson, 1998). More impressively, the Yap1 transcription factor serves as an ROS sensor for the oxidative stress response and regulates many key antioxidant genes including those that encode thioredoxin reductase (TRJ), cytosolic catalase (CTT1), thioredoxin 2 (TRX2), cytosolic superoxide dismutase (SOD1), cytochrome-c peroxidase (CCP1), and those involved in glutathione synthesis and metabolism (GSH1, GTT1, and GPX2) (Dumond et al., 2000; Moye-Rowley, 2002; Moye-Rowley et al., 1989).

Oxidative DNA damage is primarily repaired by the base excision repair (BER) pathway (Girard and Boiteux, 1997). Three yeast genes coding for DNA glycosylases, namely, OGG1, NGT1, and NTG2, have been identified, and their products catalyze the excision of oxidized bases in Saccharomyces cerevisiae. Ogg1 possesses a DNA glycosylase activity that excises 8-oxoguanine (8-OxoG) in damaged DNA, and 8-OxoG is a major base lesion produced by ROS (Thomas et al., 1997). Ntg1 and Ntg2 are N-glycosylase/apurinic/apyrimidinic (AP) lyases that remove oxidized pyrimidines from DNA (Meadows et al., 2003). The resulting abasic sites are further processed by AP endonucleases followed by long-patch or short-patch BER. The yeast APN1 and APN2 encode two class-II AP endonucleases that play redundant roles in the repair of DNA base damage (Unk et al., 2001).

It is deemed important to detect environmental pollutants that cause DNA oxidative damage at physiologically relevant doses. The RNR3-lacZ genotoxicity testing system was developed based on the fusion of DNA damage-inducible expression of RNR3 encoding a large subunit of ribonucleotide reductase in...
**YAP1 DELETION ENHANCES GENOTOXICITY**

*S. cerevisiae* with the lacZ reporter gene. This testing system is able to detect a broad range of DNA-damaging agents including oxidative agents (Jia et al., 2002). In order to enhance the detection sensitivity of the RNR3-lacZ system, we have created and characterized several deletion mutant strains including those deficient in DNA repair pathways (Jia and Xiao, 2003), cell wall components (Zhang et al., 2008), or cell wall permeability (Zhang et al., 2010). Although these manipulations significantly enhanced the RNR3-lacZ genotoxicity testing system to a variety of DNA-damaging agents, we noticed that some model oxidative compounds, such as tert-butyl hydroperoxide (t-BHP), are still poorly or not detected by the RNR3-lacZ genotoxicity testing system. Indeed, it was reported that the yeast RAD54-GFP DNA repair reporter assay (GreenScreen assay) also has poor ability to detect genotoxicity of some oxidative agents (Walsch et al., 2005). We suspected that the quick elimination of ROS by the powerful antioxidant system or the repair of the oxidative DNA damage probably account for the failure to detect genotoxicity of oxidative agents by these yeast genotoxicity testing systems.

In this study, we systematically examined the effects of yeast mutations defective in antioxidant or BER pathways on enhancement of RNR3-lacZ detection sensitivity. We report here that inactivation of YAP1 significantly sensitized yeast cells to not only oxidative damage but also other types of DNA damage. Hence, YAP1 serves as a central transcriptional regulator for DNA damage response and its singular inactivation or in combination with other genetic manipulations could massively improve the ability of yeast genotoxicity testing systems.

**MATERIALS AND METHODS**

**Yeast strains, plasmids, and transformation.** Haploid *S. cerevisiae* strains used in this study are listed in Supplementary table S1. All the strains are the isogenic derivatives of either BY4741 or SJR751. The BY4741 gene deletion derivatives were created by the Saccharomyces Gene Deletion Project and were purchased, and the *tp1Δ* strain was used as a wild-type control in this study. The identity of each deletion strain was confirmed by PCR amplification of genomic DNA using primers designed by Saccharomyces Genome Database (http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html).

To create YAP1 deletion strains, a plasmid containing the yap1Δ:hisG-URA3-hisG disruption cassette was constructed. A 2.5-kb yeast genomic DNA fragment, containing the YAP1 open reading frame (ORF) plus 0.2-kb upstream and 0.35-kb downstream sequences, was PCR amplified and cloned into pGEM-T (Promega). A 1.8-kb BamHI-BstEII fragment within the YAP1 ORF was deleted and replaced with a BamHI linker. A 3.8-kb BamHI-BsgII hisG-URA3-hisG fragment isolated from pNK51 (Alani et al., 1987) was then inserted into the above BamHI site to form pyap1Δ:hisG-URA3-hisG. The yap1Δ:hisG-URA3-hisG disruption cassette was released by AflII-EcoRI digestion. Once the yap1Δ strain was confirmed, the mutant cells were allowed to grow nonselectively overnight and selected on a plate containing 5-fluoro-orotic acid (Boeke et al., 1984) to obtain the yap1Δ:hisG derivative.

Yeast cells were grown at 30°C in YPD (Sherman et al., 1983). Plasmid DNA was transformed into yeast cells by a modified lithium acetate protocol (Hill et al., 1991) and selected on minimal SD medium (Sherman et al., 1983). Plasmid pZZ2 (Zhou and Elledge, 1992) was obtained from Dr S. Elledge (Harvard University, Boston, MA) and utilized for the RNR3-lacZ test as previously described (Jia and Xiao, 2003; Jia et al., 2002). Transformants were streaked on a fresh selective plate before being utilized for further analysis.

**DNA damage treatment and β-galactosidase assay.** Representative oxidative chemicals t-BHP, methyl viologen (MV), and H2O2 were selected for their well-characterized mechanisms of action (Lapshina et al., 2005; Yoney et al., 1986). Other genotoxic agents used in this study, including methyl methanesulfonate (MMS), 4-nitroquinoline-N-oxide (4-NQO), and plumeycin, have been previously described (Zhang et al., 2008). All the above chemicals were purchased from Sigma-Aldrich (St Louis, MO). The β-galactosidase (β-gal) assay was performed as described (Jia and Xiao, 2004; Xiao et al., 1993). Briefly, 0.5 ml of overnight yeast culture was used to inoculate 2.5 ml of fresh SD selective medium, and incubation was continued for another 2 h. At this point, chemicals were added at the concentration indicated, and cells were incubated for another 4 h. After the incubation, 1 ml of the above unsynchronized log-phase cell suspension was used to determine cell titer by measuring OD600 nm, and the remaining cells were used for the β-gal assay. Yeast cells were precipitated by centrifugation, washed twice with sterile distilled water, and resuspended in Z buffer (60mM Na2HPO4 + 7H2O, 40mM NaH2PO4 + H2O, 10mM KCl, 1mM MgSO4 + 7H2O, and 40mM β-mercaptoethanol, pH 7.0) for the β-gal assay using ortho-nitrophenyl-β-D-galactoside as the substrate. The β-gal activity is expressed in Miller units (Guerante, 1983).

For ultraviolet (UV) treatment, cells were cultured in SD-Ura medium to initial log phase (OD600 = 0.11–0.14) and 3 ml fresh culture was placed in a 100-mm tissue culture plate (BD Falcon #353003) and exposed to 254 nm UV light in a UV cross-linker (Fisher model FB-UVXL-1000) at given doses in the dark. The cells were then collected and incubated at 30°C in the dark for another 4 h prior to the β-gal assay. For γ irradiation, the fresh cell culture was exposed to a 60Co γ-ray source at a dose rate of approximately 11.1 rad/s (100 Gy/15 min) and incubated at 30°C for another 4 h prior to the β-gal assay.

**Toxicity test.** Cell survival rates were determined as previously described (Jia et al., 2002). At the end of incubation and prior to the β-gal assay, untreated and treated cells were collected by centrifugation, diluted, and plated on YPD in duplicate. The plates were incubated at 30°C for 3 days, and the number of colonies was counted. The toxic effect is expressed as a percentage of colonies from treated samples versus untreated samples. A serial dilution plate assay (Barbour et al., 2006) was also performed as a semiquantitative assessment of cellular toxicity to DNA damage.

**Effect of vitamin C on RNR3-lacZ system sensitivity to oxidative agents.** Vitamin C was dissolved in distilled water just prior to use and added into the cell culture at the given concentration 1 h before drug treatment. Treatment with 0.25mM t-BHP or 0.02% MMS was chosen based on the observation that these doses induced the highest RNR3-lacZ expression in the yap1 single deletion mutant.

**RESULTS**

**Inactivation of YAP1 Enhances Sensitivity of the RNR3-lacZ Genotoxicity Testing System to Oxidative Agents**

Yap1, a member of the AP-1 family of transcription factors, activates the transcription of antioxidant genes in response to oxidative stress (Moye-Rowley, 2002; Temple et al., 2005), and the yap1 mutant is hypersensitive to multiple oxidative stresses, including H2O2 and compounds that alter the redox status in the cell (Lopez-Mirabal et al., 2007; Schnell et al., 1992; Wenmier et al., 1994). We created the RNR3-lacZ genotoxicity testing system in the yap1 deletion mutant and tested its sensitivity to representative oxidative agents.

t-BHP is an organic hydroperoxide and serves as a useful model compound to study oxidative cell injury including DNA...
damage (Lapshina et al., 2005). The t-BHP genotoxicity can be detected by other testing systems such as the Ames test (Edenharder and Grunhage, 2003). However, t-BHP does not induce RNR3-lacZ expression (Fig. 1A) and shows no cytotoxic effect on the wild-type strain (Fig. 1D). In yap1 mutant cells, t-BHP induced obvious expression of RNR3-lacZ compared with wild-type cells (p < 0.001). For example, after 0.25mM t-BHP treatment, the yap1 mutant displayed three- to fourfold RNR3-lacZ induction, whereas no induced expression in wild-type cells was observed (Fig. 1A). Strikingly, at a concentration of 0.0625mM t-BHP, RNR3-lacZ was already induced over twofold in the yap1 mutant. The yap1 mutant also displayed dramatically enhanced sensitivity to killing by t-BHP (p < 0.001, Fig. 1D). A linear regression analysis indicates that the yap1 null mutation \( y = 27.87x + 2.422 \) enhances the RNR3-lacZ sensitivity by more than 50-fold over wild type \( y = 0.5458x + 1.238 \).

H₂O₂ is involved in the formation of hydroxyl radicals that are highly reactive, destructive, and result in direct DNA damage (Arranz et al., 2007). The yap1 mutant displayed moderately enhanced sensitivity to H₂O₂-induced RNR3-lacZ expression \( p < 0.001 \), Fig. 1B). At a H₂O₂ concentration of 0.15mM, RNR3-lacZ–induced expression was 5.4-fold in yap1 mutant compared with 3.4-fold in the wild-type strain. The increased induction in the yap1 deletion mutant is not so obvious because the basal-level expression of RNR3-lacZ is higher in the yap1 mutant. A linear regression analysis using low-dose data indicates that the inactivation of YAP1 \( y = 80.87x + 2.788 \) enhances the RNR3-lacZ sensitivity by more than fourfold over wild type \( y = 19.71x + 1.273 \). The yap1 deletion mutant displayed enhanced sensitivity to killing by H₂O₂ \( p < 0.001 \): after treatment with 0.6mM H₂O₂, only 19% of yap1 deletion mutants survived compared with about 69.3% survival of wild-type cells (Fig. 1E).

MV, commonly called paraquat \( 1,1'-\text{dimethyl-4,4'-bipyridinium dichloride, MV} \), acts as an intracellular generator of superoxide radicals and consequently causes DNA base damage.

**FIG. 1.** Sensitivity of yap1 mutant to oxidative agents. (A–C) RNR3-lacZ assays; (D–F) cell killing experiments. (A and D) t-BHP treatment; (B and E) H₂O₂ treatment; and (C and F) MV treatment. Results are the average of at least three independent experiments with SDs. **p < 0.001. Yeast strains used (●) BY4741 trp1Δ (wild type) and (◇) BY4741 yap1Δ mutant.
Deletion of YAP1 enhances MV-induced RNR3-lacZ expression (p < 0.001, Fig. 1C). A linear regression analysis using low-dose data indicates that the yap1 mutation (y = 0.3971x + 3.22) enhances the RNR3-lacZ sensitivity by 4.6-fold over wild type (y = 0.0844x + 1.613). Furthermore, a significant (p < 0.001) increase in sensitivity to killing by MV in the yap1 mutant was observed, particularly at higher doses (Fig. 1F).

In order to address whether the observed RNR3-lacZ induction was solely because of enhanced DNA damage by the yap1 mutation, we measured CYC1-lacZ reporter gene expression in response to oxidative damage in wild-type and the yap1 mutants. The CYC1-lacZ expression was not induced by oxidative damage treatments in either wild-type or the yap1 mutant strain as measured by the β-gal assay (data not shown).

Effect of ROS Pathway Mutations on RNR3-lacZ Expression

Because Yap1 is a multifunctional transcription factor that regulates the expression of a number of antioxidant pathway genes, we wish to know whether a specific oxidative stress-responsive pathway is responsible for the observed enhancement of the RNR3-lacZ induction by oxidative agents in the yap1 mutant (Dumond et al., 2000; Moye-Rowley, 2002). We further investigated the effect of ROS pathway mutations on RNR3-lacZ sensitivity under the same conditions as used in the yap1 mutant analysis. Among all null mutants (sod1, sod2, gpx1, gpx2, gsh1, and gsh2) examined, only the gsh1 single mutant exhibited a very moderate increase in sensitivity to killing by t-BHP (Supplementary fig. S1C), whereas none of the above mutations had any noticeable effects on the RNR3-lacZ expression after treatment with t-BHP or H2O2 (Supplementary fig. S1).

Effects of BER and Other DNA Repair Pathway Mutations on RNR3-lacZ Expression

It has been previously shown that inactivation of the MAG1 gene, which encodes a 3-methyladenine (3-MeA) DNA glycosylase responsible for the BER of DNA alkylating damage, is able to enhance the RNR3-lacZ sensitivity to DNA alkylating damage (Jia and Xiao, 2003). In this study, we asked whether inactivation of genes involved in the oxidative BER pathway also enhances the RNR3-lacZ sensitivity to oxidative agents. Because of functional overlaps of genes involved in the oxidative BER pathway, we measured the RNR3-lacZ expression and cell survival in response to t-BHP or H2O2 in the ogg1 single, apn1 apn2 double, and ntg1 ntg2 double mutants. Other than the increased basal-level RNR3-lacZ expression in the apn1 apn2 and ntg1 ntg2 double mutants (Supplementary figs. S2A and S2B), no obvious enhancement of the RNR3-lacZ sensitivity to oxidative damage by BER pathway mutations was observed (Supplementary fig. S2), suggesting that manipulation of the BER pathway is not likely to be able to enhance the yeast genotoxicity testing systems.

In S. cerevisiae, other DNA repair or tolerance pathways may also be involved in the processing of DNA oxidative damage (Friedberg et al., 2006). In this study, we found that deletion of POL4, RAD1, MSH2, or REV3, representing BER/nonhomologous end joining, nucleotide excision repair, mismatch repair, and translesion DNA synthesis, respectively, did not alter the RNR3-lacZ expression in response to oxidative damage (data not shown), indicating that pathways represented by these genes do not contribute significantly to the response to oxidative DNA damage under our experimental conditions.

Inactivation of YAP1 Enhances Sensitivity of RNR3-lacZ to Other Genotoxic Agents

To ask whether the yap1 mutation specifically enhances oxidative damage–induced RNR3-lacZ sensitivity, we assessed the effects of YAP1 deletion on RNR3-lacZ expression and cellular toxicity induced by other DNA-damaging agents. In this study, we chose three well-characterized and representative compounds: the DNA alkylating agent MMS; phleomycin, which induces DNA strand breaks; and 4-NQO, which induces both bulky adducts and oxidative damage.

MMS-induced RNR3-lacZ expression in the yap1 mutant is enhanced compared with wild-type cells (p < 0.001, Fig. 2A). A linear regression analysis showed that the sensitivity to MMS was increased by 3.3-fold in the yap1 mutant (y = 0.8432x – 0.9271) over wild type (y = 0.2534x – 0.2965). The yap1 mutant displayed sensitivity to 4-NQO, and the detection sensitivity was increased by 5.4-fold in the yap1 mutant (y = 12.69x + 1.408) over the wild type (y = 2.337x + 1.175) (p < 0.001, Fig. 2B).

The phleomycin detection sensitivity was also increased by 16.5-fold in the yap1 mutant (y = 0.654x + 1.673) over the wild type (y = 0.0396x + 1.077). Remarkably, at a phleomycin concentration of 2.5 μg/ml, the induced RNR3-lacZ expression can be readily observed in the yap1 mutant, whereas phleomycin concentrations up to 10 μg/ml does not induce RNR3-lacZ expression in wild-type cells (Fig. 2C).

Correspondingly, the yap1 mutant also displayed enhanced sensitivity to killing by MMS (Fig. 2D), 4-NQO (Fig. 2E), or phleomycin (Fig. 2F) compared with wild-type cells. These observations collectively suggest that inactivation of the yeast YAP1 gene function affects cellular sensitivity to nonoxidative DNA-damaging agents.

Vitamin C Protects yap1 Cells from Oxidative Damage but Not Alkylation Damage

With the observation that the yap1 mutation sensitizes cells not only to oxidative damage but also to other types of DNA damage, one question remaining to be addressed is whether these chemicals also produce free radicals. 4-NQO undergoes metabolic activation to the ultimate carcinogen 4-hydroxyaminoquinoline 1-oxide, which is then acylated and binds to DNA, producing stable quinoline-purine monoadducts at the N-2 and C-8 positions of guanine and N-6 of adenine. It is also believed that 4-NQO undergoes a redox cycling to produce a superoxide...
anion, which can be further converted into genotoxic ROS (e.g., singlet oxygen and hydroxyl radicals) that cause modified bases and DNA strand breaks (Han et al., 2007). Phleomycin belongs to a class of antitumor drugs that damage cellular DNA through the production of free radicals as well as strand breaks (Masson and Ramotar, 1996). On the other hand, MMS is a direct acting, monofunctional alkylating agent and has not been considered as an oxidative agent (Salmon et al., 2004); however, an MMS-induced dose-dependent increase in intracellular ROS levels especially in some DNA repair-defective strains has been reported (Rowe et al., 2008; Salmon et al., 2004).

In order to critically distinguish whether YAP1 deletion specifically enhances oxidative DNA damage or it also has broad effects on other types of DNA damage as well, we attempted to reverse the phenotype of yap1 mutation through the use of an antioxidant. Vitamin C is a powerful nonenzymatic, water-soluble antioxidant and acts as a scavenger of ROS, thereby potentially protecting cells from harmful oxidative products (Padayatty et al., 2003). Yeast cells naturally lack the ability to produce vitamin C, but they can accumulate vitamin C from external sources (Branduardi et al., 2007; Saffi et al., 2006). Vitamin C is able to effectively protect yeast cells against endogenous and exogenous oxidative stress (Amari et al., 2008; Bednarska et al., 2008; Branduardi et al., 2007). In this study, we found that the addition of vitamin C immediately prior to the oxidative agent t-BHP resulted in a significant and dose-dependent increase in cell survival (Fig. 3A) and a corresponding decrease in RNR3-lacZ expression (Fig. 3B) in a yap1 deletion mutant. In the presence of 10mM vitamin C, the RNR3-lacZ expression in the yap1 mutant approached the basal level (Fig. 3B) despite the fact that cells were exposed to the optimal dose of t-BHP for RNR3-lacZ induction (Fig. 1A). In sharp contrast, vitamin C was unable to rescue the cell viability (Fig. 3C) or alter RNR3-lacZ expression (Fig. 3D) induced by MMS in the yap1 mutant. The above experimental results confirm our hypothesis that deletion of YAP1 enhances cellular sensitivity not only to oxidative damage but also to other types of genotoxic damage.

**FIG. 2.** Sensitivity of yap1 mutant to other representative genotoxic agents. (A–C) RNR3-lacZ assays; (D–F) cell killing experiments. (A and D) MMS treatment; (B and E) 4-NQO treatment; and (C and F) phleomycin treatment. Results are the average of at least three independent experiments with SDs. **p < 0.001. Yeast strains used: (○) BY4741 yap1Δ (wild type) and (△) BY4741 yap1Δ mutant.
Comparison of yap1-Enhanced RNR3-lacZ Assay with Bacterium-Based Genotoxicity Assays

In order to assess the efficacy of the RNR3-lacZ test with or without YAP1 deletion and to put it into perspective with current industry standards, we attempted to define a detection limit as testing chemical concentrations that induce a twofold increase in the β-gal assay. Based on this analysis, YAP1 deletion lowered the t-BHP detection limit by 4.5-fold, H₂O₂ by 6.3-fold, and MV by 2.6-fold (Table 1). In comparison, the Ames test failed to detect H₂O₂ and MV as mutagens despite the fact that they are potent oxidative agents and induce DNA base damage (Friedberg et al., 2006). Another well-developed SOS Chromotest based on the transcriptional response of E. coli cells to DNA damage is able to detect t-BHP and H₂O₂ but fails to detect MV as a genotoxic chemical (Eder et al., 1989; von der Hude et al., 1988). As seen in Table 1, deletion of the YAP1 gene from wild-type cells also variably reduces their detection limit to MMS, 4-NQO, and phleomycin. Because the above three chemicals represent rather different types of DNA-damaging agents, it suggests that this approach could potentially enhance the detection sensitivity to a large number of genotoxic chemicals. Furthermore, the linear regression analysis data allow us to calculate the induction of RNR3-lacZ expression as a means of measuring the effect of YAP1 deletion on the improvement of the genotoxicity assay. As revealed in Table 1, deletion of the YAP1 gene alone increased the RNR3-lacZ expression induced by different chemicals by a multiple of 3.8–13.5.

Sensitivity to Radiations and Nongenotoxic Agents in the yap1 Mutant

The RNR3-lacZ testing system not only responds to DNA damage induced by chemicals but also by radiations such as UV and γ-rays (Jia et al., 2002). To ask whether deletion of the YAP1 gene also enhances the detection sensitivity of RNR3-lacZ to the above physical agents, we treated yeast cells carrying the RNR3-lacZ plasmid with UV or γ-rays. At a dose range known to induce RNR3-lacZ expression (Jia et al., 2002), deletion of YAP1 moderately enhanced the RNR3-lacZ induction by UV (Fig. 4A), and the effect by γ-rays is more apparent (Fig. 4B), which is probably because of the fact that γ-rays produce free radicals by ionizing water to form superoxide and ·OH radicals (Friedberg et al., 2006). This observation is also consistent with a recent report (Molin et al., 2007). However, under the above induction conditions, the yap1 mutant did not display apparent sensitivity to killing by UV or γ-rays, as shown by a plate assay (Fig. 4C).

FIG. 3. The effects of vitamin C on cell survival and RNR3-lacZ detection sensitivity to oxidative and alkylating agents. (A) Cellular sensitivity to t-BHP in the presence and absence of vitamin C. (B) t-BHP-induced RNR3-lacZ expression in the presence and absence of vitamin C. (C) Cellular sensitivity to MMS in the presence and absence of vitamin C. (D) MMS-induced RNR3-lacZ expression in the presence and absence of vitamin C. (A and B) 0.25 mM t-BHP treatment; (C and D) 0.02% MMS treatment. Vitamin C concentrations are as indicated. Open bars, BY4741; solid bars, BY4741 yap1Δ. Results are the average of at least three independent experiments with SDs.
TABLE 1

Minimum Detection Limits by \(\text{RNR3-lacZ}\) and Comparison with the SOS Chromotest and Ames Test

<table>
<thead>
<tr>
<th>Chemical</th>
<th>M.W. (Dalton)</th>
<th>Detection WT</th>
<th>Limit(^a) (\text{yap1})</th>
<th>Fold increase(^b)</th>
<th>SOS Chromotest(^c)</th>
<th>Ames test(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>t-BHP</td>
<td>90</td>
<td>49</td>
<td>10.9</td>
<td>9.7</td>
<td>+</td>
<td>100(^e) (TA100)</td>
</tr>
<tr>
<td>(\text{H}_2\text{O}_2)</td>
<td>34</td>
<td>37.7</td>
<td>5.98</td>
<td>13.5</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>MV</td>
<td>257</td>
<td>7770</td>
<td>2,970</td>
<td>5.9</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MMS</td>
<td>110</td>
<td>33.41</td>
<td>16.09</td>
<td>3.8</td>
<td>+</td>
<td>100 (TA100)</td>
</tr>
<tr>
<td>4-NQO</td>
<td>190</td>
<td>0.082</td>
<td>0.0058</td>
<td>4.1</td>
<td>+</td>
<td>0.125 (TA98)</td>
</tr>
<tr>
<td>Phleomycin</td>
<td>1526</td>
<td>25.08</td>
<td>7.97</td>
<td>6.3</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

\(^a\)Minimum chemical dose (in micrograms per milliter) required to cause a twofold increase in the \(\text{RNR3-lacZ}\) assay as calculated by linear regression analyses.

\(^b\)Based on the linear regression data calculated from Figures 1 and 2.

\(^c\)The SOS Chromotest data are obtained from the Pasteur Institute database

\(^d\)The Ames test data are obtained from the National Toxicological Program genotoxicity database: http://ntp-apps.niehs.nih.gov/ntp_tox/index.cfm?

\(^e\) Ames test values are based on the chemical dose (micrograms per plate) capable of inducing twofold increase in His\(^+\) revertants.

\(^f\)Based on an \(E.\ coli\) Tpr\(^+\) reversion assay (Hall, 1985).

To ask whether Yap1 protects cells from killing by chemicals that are cytotoxic but not genotoxic, we examined cell survival and \(\text{RNR3-lacZ}\) expression following tetracycline treatment. \(\text{RNR3-lacZ}\) was not induced by tetracycline in either wild-type or \(\text{yap1}\) mutant cells (Fig. 4D) despite the fact that the treatment killed up to 90% of cells (Fig. 4E). Hence, it appears that Yap1 specifically protects yeast cells from chemical-induced DNA damage.

Combined Effects of \(\text{yap1}\) with Cell Permeability Mutations

Our laboratories recently reported the enhancement of cellular sensitivity to DNA-damaging agents by inactivating the cell wall mannoproteins (Zhang et al., 2008) or the membrane efflux transporters (Zhang et al., 2010). This prompted us to investigate the possibility that Yap1 and the above cell permeability proteins jointly protect cells from oxidative damage. To address this possibility, we deleted \(\text{YAP1}\) in a quadruple mutant strain lacking \(\text{CWP1, CWP2, PDR5, and SNQ2}\) genes. To our surprise, despite the fact that the above cell permeability mutations significantly enhanced the \(\text{RNR3-lacZ}\) detection sensitivity to a broad range of DNA-damaging agents, it appears that they had no (e.g., \(t = \text{BHP or H}_2\text{O}_2\), Figs. 5A and 5B) or very moderate (e.g., Fig. 5C) effects on the \(\text{RNR3-lacZ}\) expression induced by oxidative agents, which is in sharp contrast to the \(\text{yap1}\) mutation (Fig. 5). This probably reflects the difference in molecular weight; whereas MV is considered an intermediate size molecule, \(t-BHP\) and \(\text{H}_2\text{O}_2\) are clearly small molecules. Nevertheless, inactivation of \(\text{YAP1}\) together with the four permeability genes only slightly enhanced the detection sensitivity to all three oxidative agents examined in this study (Figs. 5A–C).

We also examined the combined effects of \(\text{yap1}\) with cell permeability mutations on cellular sensitivity to MMS, 4-NQO, and phleomycin. It was interesting to note that the \(\text{yap1}\) single gene mutant is comparable (e.g., 4-NQO or phleomycin) or even more sensitive (e.g., MMS) to DNA damage than the cell permeability quadruple mutants (Fig. 6). It was surprising to observe that the combined quintuple mutation only had moderately enhanced \(\text{RNR3-lacZ}\) detection sensitivity. Nevertheless, we did observe a linear \(\text{RNR3-lacZ}\) response to phleomycin treatment at extremely low doses when neither the \(\text{yap1}\) nor the quadruple mutant showed enhanced sensitivity (Fig. 6C).

DISCUSSION

Genotoxicity testing systems including the \(\text{RNR3-lacZ}\) system based on reporter gene induction in yeast cells have shown poor sensitivity to oxidative agents (Cahill et al., 2004; Walsh et al., 2005). We suspected that the complex yeast antioxidant system effectively reduces ROS produced by oxidative agents, which can cause DNA damage and consequently induce the \(\text{RNR3-lacZ}\) expression. In order to test this hypothesis and search for a means of enhancing the detection sensitivity to oxidative damage, we systematically examined yeast mutant strains lacking individual antioxidant genes, and this approach allowed us to identify \(\text{YAP1}\) as a critical player in the protection of yeast cells from oxidative damage, whereas deletion of other related genes did not result in an obvious induction of the \(\text{RNR3-lacZ}\) genotoxicity testing system in response to oxidative agents. This is probably because of overlapping functions of these individual genes. Interestingly, despite the fact that many oxidative agents, including those examined in this study, have been known to induce DNA damage that causes cancer, premature aging, and other diseases, genotoxicity tests based on bacterial cells often failed to detect them as mutagens. The powerful antioxidant systems in bacterial cells may be responsible; hence, it remains critical to identify genes functionally similar to \(\text{YAP1}\) in order to enhance the sensitivity of genotoxicity tests such as the Ames test or SOS Chromotest.

Perhaps, the most surprising discovery made in this study is that deletion of \(\text{YAP1}\) enhances the \(\text{RNR3-lacZ}\) detection
FIG. 4. Sensitivity of yap1 mutant to physical and cytotoxic agents. (A) RNR3-lacZ expression after UV irradiation. (B) RNR3-lacZ expression after γ-ray treatment. (C) Cellular sensitivity to UV and γ-ray radiations by a 10-fold serial dilution plate assay. The isogenic rad1Δ and rad52Δ strains were used as controls for nucleotide excision repair and homologous recombination mutants, respectively. (D) RNR3-lacZ expression after tetracycline treatment. (E) Cell viability after tetracycline treatment. Yeast strains used: (●) BY4741 trp1Δ (wild type) and (□) yap1Δ mutant.
sensitivity not only to oxidative agents but also to a broad range of other DNA-damaging agents. Although we cannot definitively rule out other possibilities, results obtained in this study lend strong support to the above conclusion. First, the yap1 mutation strongly enhances the RNR3-lacZ sensitivity in terms of both maximum induction and minimum concentration of all three tested nonoxidative agents. Second, the level of above enhancement by YAP1 deletion is rather dramatic, which cannot be explained by the potential small fraction of ROS produced by some of these chemicals. Third, deletion of YAP1 also increases cellular sensitivity to killing by these nonoxidative chemicals, suggesting that Yap1 protects cells from a broad range of DNA damage. Fourth, we have previously demonstrated that deletion of several yeast genes involved in oxidative BER does not affect cellular sensitivity to MMS (Jia and Xiao, 2003), suggesting that MMS does not directly or indirectly produce ROS. Finally, although vitamin C can effectively reverse the genotoxicity and RNR3-lacZ expression induced by t-BHP, this ROS scavenger has no effect on MMS-induced genotoxicity or RNR3-lacZ expression, suggesting that this is not because of oxidative damage.

This finding is of particular interest as it pertains to our search for a means of enhancing the sensitivity of yeast genotoxic testing systems. In this case, deletion of the YAP1 gene not only allows a dramatic improvement in detecting oxidative agents but also drastically increases the detection sensitivity to other types of DNA-damaging agents with different modes of actions to a level comparable, or even superior, to the simultaneous inactivation of four cell permeability genes (Zhang et al., 2008). Overall, it is impressive that a single gene deletion in yeast cells results in an enhancement to the RNR3-lacZ detection sensitivity that is comparable or even superior to the gold standards Ames test and SOS Chromotest.

The molecular mechanisms by which Yap1 serves to restrict cellular response to DNA damage are unclear. It has been reported that most single mutants of ROS-responsive pathways displayed an increased sensitivity to oxidative stress (Luikenhuis et al., 1998; Thorpe et al., 2004). Although we did not observe the same effects, it is reasonable to suspect that the lower concentration and shorter treatment time by the oxidative agents in our study compared with previous studies account for the

![FIG. 5. Relative sensitivity of yap1 and cell permeability mutants to oxidative agents. (A–C) RNR3-lacZ assays; (D–F) cell killing experiments. (A and D) t-BHP treatment; (B and E) H2O2 treatment; and (C and F) MV treatment. Results are the average of at least three independent experiments with SDs. **p < 0.001 over wild-type cells. Yeast strains used: (○) BY4741 trp1Δ (wild type); (□) BY4741 yap1Δ (snq2Δ pdr5Δ cwp1Δ cwp2Δ); and (▲) (snq2Δ pdr5Δ cwp1Δ cwp2Δ yap1Δ).](https://academic.oup.com/toxsci/article-abstract/120/2/310/1668229)
different outcomes. Alternatively, Yap1 has been linked to several antioxidant systems and may play a central role in coordinating the cellular antioxidant responses. By the same token, Yap1 may play similar roles in regulating certain DNA repair pathway genes distinct from oxidative BER. For example, deletion of $\text{MAG1}$, encoding a 3-MeA DNA glycosylase for the first step of alkylation BER, enhances cellular detection sensitivity to MMS (Jia and Xiao, 2003). One can imagine that if Yap1 were involved directly or indirectly in the positive regulation of $\text{MAG1}$, deletion of $\text{YAP1}$ could potentially mimic the phenotypes of $\text{mag1}$. Alternatively, Yap1 may protect cells from endogenous DNA damage, particularly oxidative damage. In the absence of Yap1 activity, endogenous DNA damage may deplete the cellular DNA repair capacity, particularly BER so that cells become more susceptible to other types of DNA damage primarily repaired by BER. Some observations lend support to this argument. First, it has been previously reported that deletion of $\text{YAP1}$ enhances spontaneous mutagenesis (Huang et al., 2003) and gross chromosome rearrangement (Myung et al., 2001). Second, in this study, we noticed that deletion of $\text{YAP1}$ increased basal-level $\text{RNR3-lacZ}$ expression indicative of increased endogenous DNA damage. Furthermore, it also remains possible that Yap1 protects cells from exogenous DNA damage by regulating cell permeability genes, which could explain why the $\text{yap1}$ mutation does not have a synergistic effect with cell permeability mutations.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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