The Role of Yeast DNA 3'-Phosphatase Tpp1 and Rad1/Rad10 Endonuclease in Processing Spontaneous and Induced Base Lesions*

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Tpp1 is a DNA 3'-phosphatase in Saccharomyces cerevisiae that is believed to act during strand break repair. It is homologous to one domain of mammalian polynucleotide kinase/3'-phosphatase. Unlike in yeast, we found that Tpp1 could confer resistance to methylmethane sulfonate when expressed in bacteria that lack abasic endonuclease/3'-phosphodiesterase function. This species difference was due to the absence of 3'-lyase activity in S. cerevisiae, since expression of bacterial Fpg conferred Tpp1-dependent resistance to methylmethane sulfonate in yeast lacking the abasic endonucleases Apn1 and Apn2. In contrast, β-only lyases increased methylmethane sulfonate sensitivity independently of Tpp1, which was explained by the inability of Tpp1 to cleave 3'-α,β-unsaturated aldehydes. In parallel experiments, mutations of TPP1 and RAD1, encoding part of the Rad1/Rad10 3'-flap endonuclease, caused synthetic growth defects in yeast strains lacking Apn1. In contrast, Fpg expression led to a partial rescue of apn1 apn2 rad1 synthetic lethality by converting lesions into Tpp1-cleavable 3'-phosphates. The collected experiments reveal a profound toxicity of strand breaks with irreparable 3' blocking lesions, and extend the function of the Rad1/Rad10 salvage pathway to 3'-phosphates. They further demonstrate a role for Tpp1 in repairing endogenously created 3'-phosphates. The source of these phosphates remains enigmatic, however, because apn1 tpp1 rad1 slow growth could be correlated with neither the presence of a yeast 6-lyase, the activity of the 3'-phosphate-generating enzyme Tdp1, nor levels of endogenous oxidation.

The genomes of living cells suffer a great variety of chemical insults, with the greatest quantitative burden caused by endogenously produced DNA-damaging agents and spontaneous chemical processes (reviewed in Refs. 1, 2–6). These latter insults induce DNA lesions of principally two types: base lesions and single-strand breaks (Fig. 1). Spontaneous processes include cytosine deamination, resulting in a G:U mismatch, and spontaneous depurination, resulting in formation of an abasic site. Reactive oxygen species (ROS) are the predominant endogenous DNA damaging agent, being generated by mitochondrial respiration and other oxygen-dependent enzymatic reactions (1, 2, 7, 8). ROS oxidize bases (e.g. to 8-oxoguanine and thymine glycol) or directly cause strand breakage. Such strand breaks are typically “dirty,” having chemical moieties at their termini other than 3'-hydroxyl and 5'-phosphates. These moieties, including 3'-phosphates, 3'-aldehydes, 3'-phosphoglycolates, and 5'-deoxyribose phosphates (5'-dRP), prevent polymerization and ligation and are thus referred to as blocking lesions. All of these endogenous events can be mimicked and accelerated by exposure to select exogenous DNA damaging agents. For example, hydrogen peroxide (H2O2) induces oxidative lesions, while methylmethane sulfonate (MMS) leads to base alkylation and subsequent abasic site formation.

Several enzyme families act at base lesions and single-strand breaks, collectively forming the base excision repair (BER) pathways depicted in Fig. 1 (reviewed in Refs. 1, 3–6). Individual enzymes frequently only interconvert one form of DNA damage to another. It is through their concerted action that repair is achieved, and there is often more than one sequence leading to repair. Glycosylases remove modified bases to leave abasic sites (9, 10), such as the Mag1 enzyme of Saccharomyces cerevisiae that cleaves alkylated bases (11). A subset of glycosylases, referred to as glycosylase/lyases, additionally cleave the resulting abasic site by a non-hydrolytic β-elimination, or lyase, reaction to leave a 3' α,β-unsaturated aldehyde. β-Lyases include bacterial endonuclease III (Nth), and S. cerevisiae Ntg1, Ntg2, and Ogg1 (9, 10, 12). The more common form of BER entails hydrolytic cleavage 5' to an abasic site by two distinct but functionally similar classes of apurinic/apyrimidinic (i.e. abasic) endonucleases that leave a 3'-hydroxyl and 5'-dRP. These include bacterial endonuclease IV (Nfo) and its yeast homologue Apn1 (15), and bacterial exonuclease VIII (Nei), and recently identified human homologues (9, 10, 13, 14). The more common form of BER entails hydrolytic cleavage 5' to an abasic site by two distinct but functionally similar classes of apurinic/apyrimidinic (i.e. abasic) endonucleases that leave a 3'-hydroxyl and 5'-dRP. These include bacterial endonuclease IV (Nfo) and its yeast homologue Apn1 (15), and bacterial exonuclease VIII (Nei), and recently identified human homologues (9, 10, 13, 14). The more common form of BER entails hydrolytic cleavage 5' to an abasic site by two distinct but functionally similar classes of apurinic/apyrimidinic (i.e. abasic) endonucleases that leave a 3'-hydroxyl and 5'-dRP. These include bacterial endonuclease IV (Nfo) and its yeast homologue Apn1 (15), and bacterial exonuclease VIII (Nei), and recently identified human homologues (9, 10, 13, 14). The more common form of BER entails hydrolytic cleavage 5' to an abasic site by two distinct but functionally similar classes of apurinic/apyrimidinic (i.e. abasic) endonucleases that leave a 3'-hydroxyl and 5'-dRP. These include bacterial endonuclease IV (Nfo) and its yeast homologue Apn1 (15), and bacterial exonuclease VIII (Nei), and recently identified human homologues (9, 10, 13, 14). The more common form of BER entails hydrolytic cleavage 5' to an abasic site by two distinct but functionally similar classes of apurinic/apyrimidinic (i.e. abasic) endonucleases that leave a 3'-hydroxyl and 5'-dRP. These include bacterial endonuclease IV (Nfo) and its yeast homologue Apn1 (15), and bacterial exonuclease VIII (Nei), and recently identified human homologues (9, 10, 13, 14). The more common form of BER entails hydrolytic cleavage 5' to an abasic site by two distinct but functionally similar classes of apurinic/apyrimidinic (i.e. abasic) endonucleases that leave a 3'-hydroxyl and 5'-dRP. These include bacterial endonuclease IV (Nfo) and its yeast homologue Apn1 (15), and bacterial exonuclease VIII (Nei), and recently identified human homologues (9, 10, 13, 14).

*T The abbreviations used are: ROS, reactive oxygen species; dRP, deoxyribose phosphate; MMS, methylmethane sulfonate; H2O2, hydrogen peroxide; BER, base excision repair; NBF, nucleotide excision repair; PNKP, polynucleotide kinase/3'-phosphatase; GST, glutathione S-transferase.
cle families also cleave essentially all 3’ blocking lesions through the same active site, an activity named 3’-phosphodiesterase (17, 18). Finally, although the 3’-flap endonuclease Rad1/Rad10 is best known for its functions in nucleotide excision repair (NER) (19) and trimming nonhomologous ends during recombination (20), recent studies have suggested a role for Rad1/Rad10 in repair of strand breaks with 3’-termini (21–23).

DNA 3’-phosphatase is a more recently discovered BER enzyme. We have identified and begun to characterize Tpp1, the S. cerevisiae phosphatase (24, 25). Tpp1 is homologous to one portion of the bifunctional enzyme polynucleotide kinase/3’-phosphatase (PNKP) found in Schizosaccharomyces pombe and metazoans (26–29). It is also homologous to a family of plant enzymes, including characterized Zea mays and Arabidopsis thaliana counterparts (30, 31). Bacteria, in contrast, lack a related DNA 3’-phosphatase. Like the plant enzymes, Tpp1 is devoid of a polynucleotide kinase domain, and we have provided functional evidence that budding yeast in fact do not possess this activity (24). Thus, only the 3’-phosphatase function of this enzyme family is consistently conserved, and only in eukaryotes. Tpp1 is functionally redundant with yeast Apn1 and Apn2, which are themselves capable of removing 3’-phosphates (17, 18). A deficiency of 3’-phosphatase repair is only detected when APN1 and TTP1 both are mutated, with the greatest deficiency caused by simultaneous apn1 apn2 tpp1 deletion (25). Unlike Apn1 and Apn2, however, Tpp1 activity appears restricted to 3’-phosphates (25).

Here, we have used a variety of methods to explore the cellular function of Tpp1. We find that Tpp1 can function in bacteria to cleave 3’-phosphates left by δ-elimination, but that budding yeast lack a δ-lyase to account for the conservation of Tpp1. Tpp1 functions in the repair of endogenous DNA damage, but this effect is not realized until a redundant Rad1/Rad10 salvage pathway is also disabled. This and other results demonstrate that strand breaks with persistent irreparable blocking lesions are highly cytotoxic. The source of the requisite endogenously created 3’-phosphates remains enigmatic, but does not appear related to ROS.

**Fig. 1. Pathways for repair of abasic sites and single-strand breaks.** The drawing represents a synthesis of data from the literature and this report. Protein names used are from S. cerevisiae, but note that S. cerevisiae does not possess a δ-lyase (indicated by dashed arrow). For simplicity, 5’-end designations are only shown on the DNA duplex containing an abasic site (top); all other duplexes have the same relative strand polarities. The question mark indicates a putative unknown source of 3’-phosphates suggested by this study. See text for further discussion. Abbreviations are: CHO, 3’-α-unaslatable aldehyde; P, 3’-phosphate.
were harvested, washed twice with M9 buffer, and the cell pellet stored.

In vitro incorporation of

To measure the incorporation of

The reaction was started when

incubated with the DNA for 20 min at 37

incubation at 30

reaction was stopped with formamide/EDTA loading buffer, and samples were electrophoresed on 7 % urea, 12 % polyacrylamide gels followed by autoradiography.

TABLE I

Yeast strains used in this study

| Strain   | Genotype       |
|----------|----------------|
| YW465    | MATα ade2Δα his3Δ200 leu2 met15Δ0 trp1Δ63 ura3Δ0 |
| YW781    | MATα apn1Δ HIS3 apn2Δ:kanMX4 |
| YW857    | MATα apn1Δ HIS3 apn2Δ:kanMX4 |
| YW950/951| MATα apn1Δ HIS3 apn2Δ:kanMX4 tpp1Δ:MET15 |
| YW952    | MATα can1Δ Myc-Fpg apn1Δ HIS3 apn2Δ:kanMX4 |
| YW959/966| MATα can1Δ Myc-Fpg apn2Δ:kanMX4 |
| YW963    | MATα can1Δ Myc-Fpg apn2Δ:kanMX4 |
| YW851/1014| MATα-incMATα APN1:nop1Δ:HIS3 APN2:nop2Δ:kanMX4 TPF1:tpplΔ:MET15 RAD1:rad1Δ:URA3 |
| YW1035   | MATα-incMATα APN1:nop1Δ:HIS3 APN2:nop2Δ:kanMX4 |
| YW1012   | MATα-incMATα COQ3:nop3Δ:kanMX4 APN1:nop1Δ:HIS3 APN2:nop2Δ:kanMX4 TPF1:tpplΔ:MET15 |
| YW1013   | MATα-incMATα COQ3:nop3Δ:kanMX4 APN1:nop1Δ:HIS3 APN2:nop2Δ:kanMX4 TPF1:tpplΔ:MET15 RAD1:rad1Δ:URA3 |
| YW1055   | MATα-incMATα COQ3:nop3Δ:kanMX4 APN1:nop1Δ:HIS3 APN2:nop2Δ:kanMX4 TPF1:tpplΔ:MET15 RAD1:rad1Δ:URA3 |
| YW1033   | MATα-incMATα TDIP1:tdplΔ:kanMX4 APN1:nop1Δ:HIS3 APN2:nop2Δ:kanMX4 TPF1:tpplΔ:MET15 |
| YW1042   | MATα-incMATα TDP1:tdplΔ:kanMX4 APN1:nop1Δ:HIS3 APN2:nop2Δ:kanMX4 |
| YW1097   | MATα-incMATα CAN1:nop1Δ:Fpg APN1:nop1Δ:HIS3 apn2Δ:kanMX4 apn2Δ:kanMX4 |
| YW908    | MATα-incMATα CAN1:nop1Δ:Fpg APN1:nop1Δ:HIS3 apn2Δ:kanMX4 apn2Δ:kanMX4 TPF1:tpplΔ:MET15 RAD1:rad1Δ:URA3 |

For clarity, common marker alleles have been omitted from all strains except YW465. Thus, all other strains are additionally ade2Δα his3Δ200 leu2 met15Δ0 trp1Δ63 ura3Δ0 (homozygous for diploids strains).

**RESULTS**

**Tpp1 Can Participate in Repair of H2O2 and MMS-induced Lesions When Expressed in Bacteria—*E. coli* strain BW528 bears mutations in both the *nfo* and *xth* abasic endonuclease genes and is consequently hypersensitive to agents that induce abasic sites or strand breaks. Because this strain has proven useful for the study of a number of heterologously expressed BER proteins (29, 30, 37), we examined whether a GST-Tpp1 fusion protein could complement the BW528 mutations. In our first assay, the bacterial chromosome is damaged in *vivo* with *H2O2* and subsequently isolated and used as a template for primer extension by DNA polymerase I *in vitro* (37). Only if 3′-blocking lesions are removed at *H2O2*-induced strand breaks can ['H]dTMP be incorporated. Thus, DNA isolated from the treated wild-type strain AB1157 supported primer extension without pretreatment (Fig. 2A), while DNA isolated from BW528 supported extension only when it was pretreated with enzymes such as Nfo (data not shown, but see Tpp1-D35A mutation, Fig. 2C and below). In contrast, DNA from BW528 expressing GST-Tpp1 supported substantial primer extension without pretreatment (Fig. 2B). The amount of ['H]dTMP incorporation could be increased to a minor extent by pretreatment of this DNA with Nfo, but not with pretreatment with hPNK. Thus, GST-Tpp1 expressed in *vivo* was able to remove most, but not all, *H2O2*-induced 3′-blocking lesions. Since *H2O2* leaves principally, but not exclusively, 3′ phosphates (38, 39) (see Fig. 1), these data support the interpretation that the activities of Tpp1 and hPNK are similar and restricted to 3′-phosphates.

Examing the survival of bacteria treated with *H2O2* gave similar results, but not completely, rescure survival of BW528 as compared with wild type (Fig. 3A). This result parallels previous experiments in yeast showing that Tpp1 confers *H2O2* resistance (25). We were initially surprised to see that GST-Tpp1 could also confer partial BW528 resistance to MMS, however (Fig. 3B). This was unexpected since MMS leads principally to the formation of abasic sites through the action of DNA glycosylases (see Fig. 1), not 3′-phosphates. Indeed, *apn1 apn2 tpp1* triple mutant yeast are no more sensitive to MMS than *apn1 apn2* double mutant yeast (25). In the following experiments we sought to determine the basis of the differing effects of Tpp1 expression on MMS sensitivity in yeast and bacteria.
**Tpp1 Cannot Remove 3’-Aldehydes**—One explanation for the bacterial result would be that Tpp1 in fact has a 3’-phosphodiesterase activity, beyond 3’-phosphatase, that is able to cleave a subset of blocking lesions created by MMS treatment. We have previously shown that Tpp1 can not cleave abasic sites or 3’-phosphoglycolates, nor is it an exonuclease (25). An untested high frequency 3’-blocking lesion was the unsaturated aldehyde resulting from elimination at a deoxyribose. As shown in Fig. 4, GST-Tpp1, unlike GST-Apn1, showed no activity at this lesion, even at enzyme concentrations 40-fold higher than those required to completely cleave a similar amount of 3’-phosphates. It is therefore highly likely that the beneficial effect of Tpp1 in bacteria is explainable purely by its 3’-phosphatase activity.

**Fpg, but Not Nth, Confers Tpp1-dependent Phenotypes in apn1 apn2 Yeast**—Our preferred hypothesis for the unexpected rescue of BW528 MMS resistance by GST-Tpp1 relates to the secondary metabolism of abasic sites (see Fig. 1). Specifically, action of the bacterial δ-lyases Fpg and Nei at MMS-induced abasic sites would produce 3’-phosphates that are a suitable substrate for GST-Tpp1. In contrast, yeast are not known to express a δ-lyase, although the recent unanticipated demonstration of Nei-related enzymes in humans makes this claim uncertain (13, 14). We reasoned that if the difference between the bacterial and yeast MMS experiments was indeed a result of δ-lyase expression, then yeast should become more like bacteria if a δ-lyase was expressed ectopically.

Our goal was to express the *E. coli fpg* gene in *S. cerevisiae*. 
This experiment was complicated by the fact that the amino-terminal proline of Fpg acts as the Schiff base nucleophile during the sequential $\beta$- and $\delta$-elimination reactions (40). Consequently, even a two residue (Gly-Met) N-terminal extension impairs Fpg activity by more than 100-fold (41). The extent to which the obligatory initiator methionine would be removed in yeast to leave an N-terminal proline could not be predicted, but fpg is known to complement ogg1 mutation in S. cerevisiae, indicating that some function is retained (42). We attempted to express Fpg both with and without an amino-terminal Myc tag.

Fig. 5A and Table II show the interesting result that only very poorly growing colonies were obtained when untagged Fpg was expressed in apn1 apn2 tpp1 yeast. In contrast, Fpg was entirely tolerated by apn1 apn2 yeast. Although these results largely precluded meaningful drug sensitivity testing with untagged Fpg, they already demonstrated that Fpg could confer a Tpp1-dependent phenotype in yeast, presumably by cleaving spontaneously arising DNA lesions (see more below). Myc-Fpg could be expressed in any yeast strain (Fig. 5A), indicating that it is less active than untagged Fpg, as expected. We reasoned that Myc-Fpg might nonetheless retain sufficient lyase activity to satisfy our initial goal of conferring Tpp1-dependent MMS resistance on yeast. This was indeed the case (Fig. 5B).

apn1 apn2 and apn1 apn2 tpp1 yeast were themselves highly and equivalently sensitive to MMS, as previously demonstrated (25). Expression of Myc-Fpg in the apn1 apn2 background led to a measurable increase in resistance, although well below wild-type even at the low (0.01–0.1%) MMS concentrations used (compare with the apn2 strain in Fig. 5B). This effect was entirely dependent on Tpp1, the only remaining 3'-phosphatase in the cells, demonstrating that it was due to Myc-Fpg-dependent $\delta$-elimination. In fact, Myc-Fpg dramatically increased the MMS sensitivity of apn1 apn2 tpp1 yeast, such that cultures were effectively sterilized by a 30 min treatment with the extremely low MMS concentration of 0.025%. Tpp1-dependent MMS resistance was also seen when Myc-Nei was heterologously expressed.2

The implication of the above results is that Myc-Fpg was able which the obligatory initiator methionine would be removed in yeast to leave an N-terminal proline could not be predicted, but fpg is known to complement ogg1 mutation in S. cerevisiae, indicating that some function is retained (42). We attempted to express Fpg both with and without an amino-terminal Myc tag.

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2 A. S. Karumbati, R. A. Deshpande, A. Jilani, J. R. Vance, D. Ramotar, and T. E. Wilson, unpublished observations.
to convert less toxic abasic lesions to strand breaks that became highly toxic in the absence of enzymes able to remove 3'-phosphate blocking lesions. This would predict that (over)expression of a β-only lyase would lead to an increase in MMS killing regardless of the presence of Tpp1, since Tpp1 cannot remove 3'-aldehydes (see above). Fig. 5C shows that Myc-Nth did indeed lead to an equivalent increase in MMS sensitivity in both \( \text{apn1 apn2} \) and \( \text{apn1 apn2 tpp1} \) yeast. A similar pattern was observed when the yeast glycosylases Ogg1, Ntg1, or Ntg2 were overexpressed as Myc fusion proteins from the strong constitutive \( \text{ADH1} \) promoter.2 These results provide clear in \( \text{vivo} \) corroboration of the lesion specificity of Tpp1.

**Tpp1 Amino Acids Asp-35 and Asp-37 Are Essential for Its in Vivo Function**—In addition to conferring H\(_2\)O\(_2\) and MMS resistance in bacteria (Fig. 3), plasmid-expressed Tpp1 reduced MMS sensitivity of \( \text{apn1 apn2} \) yeast more than 1500-fold (Fig. 5D). To validate dependence of these assays on Tpp1 catalytic function, we examined mutations of two universally conserved Tpp1 aspartate residues, Asp-35 and Asp-37. Based on homology with the L-haloacid dehalogenase superfamily of hydrolases, these residues are believed to participate in catalysis by forming a covalent bond between the phosphate-leaving group and the first aspartate (24). Mutation of either aspartate to alanine (D35A and D37A) completely abolished Tpp1-dependent repair of H\(_2\)O\(_2\)-induced lesions (Fig. 2 and data not shown) and resistance to both H\(_2\)O\(_2\) and MMS in bacteria (Fig. 3), as well as resistance to MMS in Myc-Fpg-expressing yeast (Fig. 5D). GST-Tpp1-D35A and GST-Tpp1-D37A were also completely inactive in \( \text{vivo} \).

**Synthetic Growth Defects Caused by \( \text{apn1 apn2 tpp1} \), and \( \text{rad1} \) Mutant Combinations**—We have previously seen that \( \text{apn1 apn2 tpp1} \) yeast grow similarly to wild-type but die when the recombination gene \( \text{RAD52} \) is deleted, and that \( \text{apn1 apn2 rad52} \) and \( \text{apn1 apn2 rad52 tpp1} \) yeast grow extremely poorly (25). This suggested a recombination-dependent mechanism for removal of 3'-blocking lesions. To explore this hypothesis further, we performed analogous tetrad dissection experiments with the \( \text{RAD1} \) gene, which encodes part of the Rad1/Rad10 endonuclease that acts to trim 3'-ends during recombination (20). As shown in Fig. 6, \( \text{apn1 tpp1 rad1} \) strains grew very poorly, similar to \( \text{apn1 tpp1 rad52} \). This effect was not due to the NER deficiency of \( \text{rad1} \) mutants (19) since \( \text{rad2} \) and \( \text{rad14} \) NER mutants did not share the \( \text{tpp1} \) synthetic phenotype.2 Strikingly, \( \text{apn1 apn2 rad1} \) strains showed an even more severe phenotype of synthetic lethality, similar to a recent report (23). Microscopic examination of inferred \( \text{apn1 apn2 rad1} \) microcolonies showed them to be typically comprised of 10 to ~50 large, budded, and misshapen cells (Table II and data not shown), characteristic of persistent irreparable DNA damage. When \( \text{tpp1} \) mutation was added this phenotype became even more severe, such that \( \text{apn1 apn2 tpp1 rad1} \) cells rarely formed microcolonies of more than 10 cells. The collected results demonstrate that while the synthetic interaction with \( \text{rad1} \) mutation is most severe for \( \text{apn1 apn2} \) mutation, the phenotype extends to \( \text{tpp1} \) mutation, which is informative because Tpp1 is a pure DNA 3'-phosphatase.

**Partial Suppression of the \( \text{apn1 apn2 rad1} \) Growth Defect by Fpg**—The most likely explanation for \( \text{apn1 apn2 rad1} \) synthetic
lethality is that spontaneously arising abasic sites either persist or are converted to strand breaks with 3′-aldehyde blocking lesions by β-lyases (see “Discussion”). In the absence of a δ-lyase or abasic endonuclease/3′-phosphodiesterase, these lesions would persist and require Rad1/Rad10 for removal. A prediction of this model is that ectopic expression of a δ-lyase should rescue apn1 apn2 rad1 lethality in a Tpp1-dependent fashion. Indeed, apn1 apn2 rad1 yeast expressing untagged Fpg did form visible colonies, but not when TPPI was also mutated (Table II). The rescue was partial, however, since these colonies were very small and failed to continue growing. This may be because only a small portion of the Fpg expressed in yeast is made competent for δ-elimination by removal of the amino-terminal methionine. Alternatively, apn1 apn2 rad1 lethality may depend on functions in addition to processing of abasic sites and 3′-aldehydes.

Suppression of apn1 tpp1 rad1 Synthetic Slow Growth—Extending the above logic, endogenous DNA damage must also explain the tpp1 rad1 synthetic interaction, and this damage must include 3′-phosphates. There are three known mechanisms by which 3′-phosphates are generated in growing cells. First is through the action of 3′-phosphate lyases as discussed, but the above experiments functionally demonstrate that S. cerevisiae does not possess a δ-lyase (see “Discussion”).

Second, we and others have described a pathway in which the enzyme Tdp1 removes the DNA topoisomerase Top1 when the latter is irreversibly and covalently bound to a DNA 3′-phosphate (43) that is removed by the combined action of Tpp1, Apn1, and Apn2 (see Fig. 1(25)). The slow growth phenotype of tdp1 rad1 yeast demonstrates that the Tdp1 pathway is active to some extent even in the absence of Top1 inhibitors (21). This tdp1 rad1 slow growth is much less severe than even the apn1 tpp1 rad1 phenotype, however, making it unlikely that the Top1 pathway contributes substantially to the tpp1 rad1 synthetic interaction. Indeed, mutation of neither TDP1 nor TOP1 led to a measurable suppression of the apn1 tpp1 rad1 growth defect (Table II).

Third, endogenous reactive oxygen species can lead directly to the formation of strand breaks with 3′-phosphates in the same manner as exogenous H2O2. We therefore compared the growth of apn1 tpp1 rad1 and apn1 tpp1 yeast in aerobically and anaerobically grown streaks (Fig. 7). The profound growth difference of these strains was preserved on aerobic re-streaking.

| Genotype            | Colony size* | Total |
|---------------------|-------------|-------|
|                     | 1 | 2 | 3 | 4 | 5 |       |
| Wild-type           | 7 | 21 | 28 |
| apn1                | 5 | 46 | 51 |
| apn2                | 2 | 67 | 89 |
| apn1 tpp1           | 12 | 45 | 57 |
| apn1 apn2           | 14 | 48 | 63 |
| apn2 rad1           | 90 | 90 |
| apn1 rad1           | 16 | 50 |
| apn1 tpp1 rad1      | 81 | 84 |
| apn1 apn2 rad1      | 36 | 63 |
| apn1 apn2 tpp1 rad1 | 100 | 100 |
| can1:Fpg apn1 apn2  | 39 | 39 |
| can1:Fpg apn1 apn2 tpp1 | 26 | 26 |
| can1:Fpg apn2 rad1  | 99 | 101 |
| can1:Fpg apn1 apn2 rad1 | 43 | 43 |
| tdp1 apn1 tpp1      | 8 | 16 |
| tdp1 apn1 rad1      | 14 | 22 |
| tdp1 apn1 tpp1 rad1 | 11 | 22 |
| top1 apn1 tpp1      | 16 | 16 |
| top1 apn1 rad1      | 16 | 16 |
| coq3 apn1           | 6 | 6 |
| coq3 apn2           | 5 | 5 |
| coq3 rad1           | 4 | 4 |
| coq3 apn1 rad1      | 6 | 6 |
| coq3 apn2 rad1      | 3 | 3 |
| total               | 28 | 28 |

*The size of the colonies formed after growth of tetrad dissection plates was determined prior to genotyping according to the following scoring system: 5, Wild-type colony growth (e.g. Fig. 6, A3). 4, Intermediate between 5 and 3 (e.g. Fig. 6, B3). 3, Macrocolony visible at 3 days growth (e.g. Fig. 6, A5). 2, Microcolony (not macroscopically visible at 5 days), >10 cells. 1, Inconspicuous; 0, Single cell, i.e. no germination (not tabulated).
and same media for comparison. 5-Fold lower concentrations of ascorbate dependent means, but without success. Growth in a reduced O2 therefore sought to corroborate the anaerobic findings by inde-

regardless of genotype and despite the inclusion of lipid nutri-

cells in anaerobically grown colonies were nearly all dead, 

slow growth. However, when we attempted to verify this 

rad1 

e ndogenous oxidation is a significant contributor to 

the appearance of the two strains. This initially suggested that 

mutation. In contrast, anaerobic growth tended to normalize 

i.e.

Numerous large (apn1 tpp1, colony size 5; see Table II) and small (apn1 tpp1 rad1, colony size 3) macrocolonies were picked from tetrads and streaked, and grown on the following media for the indicated number of days: YPD, 2 days; YPD plus 0.5% Tween 80 and 20 μg/ml ergosterol, grown anaerobically for 3 days; YPD plus 25 mM ascorbic acid, 2 days; YPD plus 25 mM N-acetyl cysteine, 2 days; YP medium with ethanol-glycerol as the carbon source, 5 days. Representative streaks are shown. Iso-

genic SOD1 wild-type and sod1 mutant strains were streaked to these same media for comparison. 5-Fold lower concentrations of ascorbate and N-acetylcysteine gave equivalent results for all strains.

ing, i.e. small spore colonies were not an artifact of slow ger-

ination. In contrast, anaerobic growth tended to normalize 

the appearance of the two strains. This initially suggested that endogenous oxidation is a significant contributor to apn1 tpp1 rad1 slow growth. However, when we attempted to verify this phenotype by additional rounds of restreaking, we found that cells in anaerobically grown colonies were nearly all dead, regardless of genotype and despite the inclusion of lipid nutri-

tional or enzymatic 

streaked from ethanol-glycerol back to dextrose once again 

showed severely impaired growth. Finally, we observed spontaneouly arising slow-growth suppressors in aerobic streaks of 
apn1 tpp1 rad1 yeast, but none of 18 tested proved to be respiratory (i.e. petite) mutants. The implications of these findings are addressed in “Discussion.”

DISCUSSION

The Tpp1 Family of Enzymes is 3′-Phosphate-specific—It has previously been demonstrated that both human and plant DNA 3′-phosphatases are able to restore DNA damage resistance in bacteria lacking the abasic endonucleases Nfo and Xth (29, 30). We have found this to be true for Tpp1 as well (Fig. 3). There are numerous implications of this finding. First, Tpp1 can clearly act at 3′-phosphate lesions in vivo independently of other yeast proteins. Further, the extent to which Tpp1 could restore polymerase priming capacity to H2O2-damaged DNA (Fig. 2) draws a parallel to hPNKP function. H2O2-induced damage is characterized predominantly by strand breaks with 3′-phosphates (38, 39). Tpp1 and hPNKP each facilitated ex-
tension by DNA polymerase I by removing these phosphates, but neither completely restored priming capacity as compared with the multifunctional 3′-phosphodiesterase Nfo. This indicates that Nfo, but not Tpp1 or hPNKP, is able to cleave the minority of non-oxidative H2O2 lesions as well. Supporting this interpretation, we have demonstrated that Tpp1 is inac-

ative at a wide variety of non-oxidative strand lesions, including abasic sites, simple nicks, 3′-phosphoglycolates (25), and 3′-aldehydes (Fig. 4). We have seen a similar pattern with the S. pombe PNKP homologue (27). DNA 3′-phosphatases from yeast and human thus appear to have a similar and consider-
ably more restricted function than the abasic endonucleases. This is consistent with the inferred catalytic mechanism of this enzyme family (24), which is supported by our findings that Tpp1 D35A and D37A mutants are catalytically inactive (Figs. 2, 3, and 5).

δ-Elimination Is Not Required for Genome Maintenance in S. cerevisiae—It was initially surprising that Tpp1 confronted bacterial resistance to MMS as well H2O2. This finding was best explained by the presence of lyases in bacteria that can create 3′-phosphates from MMS-induced abasic sites by consecutive δβ elimination (Fig. 1). We validated this hypothesis by demonstr-

ating that even the crippled Myc-Fpg led to a clear and predictible Tpp1-dependent MMS resistance when expressed 

in vivo 

in yeast (Fig. 5). The complete lack of Tpp1-dependent MMS resistance in the parent strains thus demonstrates functionally that S. cerevisiae does not possess a detectable δ lyase. This conclusion is supported by the fact that the characteristic structural features of the Fpg/Nei glycosylase/lyase family are not shared by any hypothetical protein in S. cerevisiae, as determined by BLAST searches or by considering all open 

reading frames with a proline as the second residue (14). It is thus apparent that yeast ordinarily maintain their genomes without the benefit of δ-elimination, and must instead rely on the abasic endonucleases to remove 3′-aldehydes left by chemi-

cal or enzymatic β-elimination. This is important in light of the recent unexpected discovery of functional homologues of Fpg/Nei in many eukaryotes, including humans and the fungus Candida albicans (13, 14).

The paradox is this: why do bacteria possess δ-lyases but no 

pure 3′-phosphatase, while yeast possess a 3′-phosphatase but 

no δ-lyase? At a minimum, this paradox makes clear that Tpp1 containing the non-fermentable carbon sources ethanol and glycerol forces respiratory metabolism. Remarkably, ethanol-glycerol medium did not exacerbate apn1 tpp1 rad1 slow growth, but in fact completely suppressed it (Fig. 7). This phenotype was reversible because apn1 tpp1 rad1 colonies streaked from ethanol-glycerol back to dextrose once again showed severely impaired growth. Finally, we observed spontaneouly arising slow-growth suppressors in aerobic streaks of 
apn1 tpp1 rad1 yeast, but none of 18 tested proved to be respiratory (i.e. petite) mutants. The implications of these findings are addressed in “Discussion.”

overlapping function of Tpp1 and Rad1/Rad10 at 3′-phosphates

Fig. 7. Suppression of the growth defect of apn1 tpp1 rad1 yeast by altering the growth conditions. Numerous large (apn1 tpp1, colony size 5; see Table II) and small (apn1 tpp1 rad1, colony size 3) macrocolonies were picked from tetrads and streaked, and grown on the following media for the indicated number of days: YPD, 2 days; YPD plus 0.5% Tween 80 and 20 μg/ml ergosterol, grown anaerobically for 3 days; YPD plus 25 mM ascorbic acid, 2 days; YPD plus 25 mM N-acetyl cysteine, 2 days; YP medium with ethanol-glycerol as the carbon source, 5 days. Representative streaks are shown. Iso-
genic SOD1 wild-type and sod1 mutant strains were streaked to these same media for comparison. 5-Fold lower concentrations of ascorbate and N-acetylcysteine gave equivalent results for all strains.
does not exist to cleave 3'-phosphate lesions left by δ-elimination. Indeed, in bacteria this need is satisfied by Nfo and Xih. The question remains why Tpp1 is ever necessary, or preferred, in yeast cells that already possess Apn1 and Apn2.

**A General Rad1/Rad10-dependent Salvage Pathway for Repair of 3'-Blocked Strand Breaks—**Our results support the conclusion that Tpp1 participates in the repair of endogenous DNA damage, but in a fashion redundant with other 3' repair mechanisms. This was not initially obvious because even apn1 apn2 tpp1 cells grow at a normal rate. However, the addition of either rad52 (25) or rad1 (Fig. 6, Table II) mutation leads to lethality in this background, suggesting that Rad52 and Rad1/Rad10 act in a redundant pathway that repairs persistent short breaks. These results parallel similar recent findings. For example, Rad1/Rad10 participates in repair of strand breaks when Top1 peptide fragments are covalently bound to the 3' terminus, but only in the absence of Tdp1, the enzyme that normally removes these peptides (21, 22). In addition, the apn1 apn2 rad1 mutation combination is lethal due to absence of Rad1/Rad10-dependent processing of 3'-aldehyde lesions that persist in apn1 apn2 cells (23). Analysis of tpp1 phenotypes is necessarily more complex, because depriving cells of 3'-phosphatase function requires apn1 and apn2 mutation and thus concomitant loss of abasic endonuclease/phosphodiesterase activity. Nonetheless, tpp1 mutation causes an incremental growth defect in both apn1 rad1 and apn1 apn2 rad1 backgrounds (Fig. 6 and Table II). The lesion specificity of Tpp1 dictates that this effect must be due to failed repair of endogenously generated 3'-phosphates, and in turn that 3'- phosphates can be repaired by the Rad1/Rad10 pathway. The capacity of this pathway is clearly limited, however, since it cannot protect apn1 apn2 tpp1 cells from higher levels of H2O2-induced damage (25). Taken together, we argue that Rad1/Rad10-dependent repair of 3'-phosphates is truly a salvage pathway not normally active in cells expressing Tpp1. Whether there is any normal function of this Rad1/Rad10 pathway of strand break repair remains to be determined.

**Sources of Endogenously Generated 3'-Phosphates—**Surprisingly, there appears to be little correlation between the endogenous mitochondrial ROS burden and the apn1 tpp1 rad1 growth defect. Anaerobic growth did normalize the growth of apn1 tpp1 rad1 strains (Fig. 7), but reducing mitochondrial ROS production by coq3 mutation did not (Table II). Instead, attempting to increase mitochondrial ROS production by growth on non-fermentable carbon sources anti-ynthetically suppressed apn1 tpp1 rad1 slow growth (Fig. 7). It is possible that the induction of the oxidative stress response that occurs during respiratory growth (48) more than compensates for the increased oxidative load. Exogenous ROS scavengers were ineffective in suppressing apn1 tpp1 rad1 slow growth, however, arguing against this hypothesis (Fig. 7).

A different explanation would be that the anaerobic environment and non-fermentable carbon sources each suppress the apn1 tpp1 rad1 growth defect independently of oxidation. Slow growth might simply allow more time for repair. Alternatively, faster replication and/or growth on glucose might itself result in increased production of 3'-phosphates. Given that S. cerevisiae does not possess a δ-lyase, the enzyme Tdp1 is the only remaining known non-oxidative source of endogenous 3'-phosphates in this organism (43). However, while Top1 damage might be exacerbated by rapid replication, the Top1-Tdp1 pathway apparently does not lead to enough basal damage to account for the apn1 tpp1 rad1 slow growth phenotype (Table II). We must therefore consider the possibility that there is an as yet unidentified intracellular source of 3'-phosphates. This would most likely be an enzyme, perhaps one under carbon source regulation. δ-Lyases and Tdp1 give clear precedent for the notion that a DNA metabolic enzyme might leave a 3'-phosphate in need of further repair.

**Persistent Strand Breaks Are Especially Cytotoxic—**Xiao et al. (11) have demonstrated that metabolism of modified bases to abasic sites is necessary to achieve cell killing and mutagenesis by agents such as MMS. A consistent implication of our studies is that it is secondary strand breaks, rather than abasic sites themselves, that are most cytotoxic. Guillet et al. (23) recently came to a similar conclusion. They demonstrated that the lethality of apn1 apn2 rad1 yeast was suppressed by the combined mutation of the β-only lyases expressed in S. cerevisiae, OGG1, NTG1, and NTG2, which suggested that it is 3'-aldehydes that are least well tolerated. We have extensively employed the complementary approach of expressing glycolyase/lyases to interconvert lesions to predictable endpoints. Overexpression of β-lyases, including Nth, Ogg1, Ntg1, and Ntg2, enhanced the already substantial MMS sensitivity of apn1 apn2 yeast (Fig. 5). In contrast, even Fpg weakened by an amino-terminal fusion was able to reduce this MMS sensitivity, presumably by converting toxic 3'-aldehydes into Tpp1-reparable 3'-phosphates (Fig. 5). Indeed, Myc-Fpg-expressing apn1 apn2 tpp1 yeast were exquisitely MMS hyper-sensitive. Similarly, untagged Fpg was able to partially suppress apn1 apn2 rad1 lethality (Table II), but conversion of even endogenous levels of base lesions to 3'-phosphates by Fpg overwhelmed the capacity of Rad1/Rad10 in the absence of 3'-phosphatases (Fig 5 and Table II).

We conclude that it is not strand breaks themselves that are toxic, but rather their persistence due to irreparable 3' blocking lesions. Replication would thus lead to the formation of double-strand breaks still bearing the blocking lesion, as we have modeled in detail for camptothecin-induced damage (21). Interestingly, this overall pattern is apparently not restricted to 3'-blocking lesions. It is the lyase activity of mammalian DNA polymerase β, which removes 5'-dRP blocking lesions, that is most critical during BER (49). In lyase-deficient mutants, 5'-blocked strand breaks persist and are thought to lead to a similar catastrophe during replication.

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The Role of Yeast DNA 3′-Phosphatase Tpp1 and Rad1/Rad10 Endonuclease in Processing Spontaneous and Induced Base Lesions
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