MOLECULAR AND CELLULAR BIOLOGY, Nov. 2001, p. 7191–7198 Vol. 21, No. 21
0270-7306/01/$04.00+0 DOI: 10.1128/MCB.21.21.7191–7198.2001

Repair of DNA Strand Breaks by the Overlapping Functions of Lesion-Specific and Non-Lesion-Specific DNA 3′ Phosphatases

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Received 11 May 2001/Returned for modification 12 June 2001/Accepted 1 August 2001

In *Saccharomyces cerevisiae*, the apurinic/apyrimidinic (AP) endonucleases Apn1 and Apn2 act as alternative pathways for the removal of various 3′-terminal blocking lesions from DNA strand breaks and in the repair of abasic sites, which both result from oxidative DNA damage. Here we demonstrate that Tpp1, a homologue of the 3′ phosphatase domain of polynucleotide kinase, is a third member of this group of redundant 3′ processing enzymes. Unlike Apn1 and Apn2, Tpp1 is specific for the removal of 3′ phosphates at strand breaks and does not possess more general 3′ phosphodiesterase, exonuclease, or AP endonuclease activities. Deletion of *TPP1* in an *apn1 apn2* mutant background dramatically increased the sensitivity of the double mutant to DNA damage caused by H_2O_2 and bleomycin but not to damage caused by methyl methanesulfonate. The triple mutant was also deficient in the repair of 3′ phosphate lesions left by Tdp1-mediated cleavage of camptothecin-stabilized Top1-DNA covalent complexes. Finally, the *tpp1 apn1 apn2* triple mutation displayed synthetic lethality in combination with *rad52*, possibly implicating postreplication repair in the removal of unrepaired 3′-terminal lesions resulting from endogenous damage. Taken together, these results demonstrate a clear role for the lesion-specific enzyme, Tpp1, in the repair of a subset of DNA strand breaks.

Reactive oxygen species (ROS) generated by the mitochondria during aerobic metabolism can induce several types of DNA damage, including abasic (apurinic/apyrimidinic [AP]) sites, base modifications, and DNA strand breaks. Strand breaks caused by ROS, as well as from exposure to ionizing radiation or treatment with the anticancer agent bleomycin, often contain unconventional terminal groups, such as 3′ phosphoglycolates (PGs) and 3′ phosphates, resulting from fragmentation of deoxyribose sugars (7). These blocking lesions must be removed to allow repair of the breaks by polymerization and ligation. AP endonucleases, which function during base excision repair (BER) of abasic sites, also exhibit 3′ phosphodiesterase activity and provide a major pathway for removal of 3′-terminal lesions (2, 12, 13, 23). Two AP endonucleases, Apn1 and Apn2, have been identified in *Saccharomyces cerevisiae*. Apn1 is a homologue of *Escherichia coli* endonuclease IV and represents the major AP endonuclease in yeast, constituting >90% of the activity in cellular extracts (12). Apn2 (also called Eth1) shares homology with *E. coli* exonuclease III and human Hap1/Ape1 and is induced after exposure to DNA-damaging agents such as the alkylating agent methyl methanesulfonate (MMS) (2, 13). Like Apn1, Apn2 possesses both AP endonuclease and 3′ phosphodiesterase activities; however, only Apn2 has been shown to have an associated 3′-5′ exonuclease activity (23). In *S. cerevisiae*, *apn1 apn2* double mutant strains display a synergistic increase in sensitivity to MMS and H_2O_2, indicating that these enzymes perform overlapping roles in the repair of abasic sites and in the repair of strand breaks with 3′-terminal lesions (2, 13).

We recently identified a DNA 3′ phosphatase in *S. cerevisiae*, *TPP1*, based on its homology to the bifunctional human enzyme polynucleotide kinase-3′ phosphatase (hPNKP) (25). hPNKP interacts with XRCC1 and is thought to function during BER–single-strand break repair by restoring normal termini to strand breaks containing 3′ phosphates and 5′ hydroxyls (26), although in vivo evidence to support this is lacking. Interestingly, Tpp1 shares significant homology with only the 3′ phosphatase domain of hPNKP. A corresponding 5′ kinase domain is entirely absent from the genome. Consistent with these observations, biochemical characterization of Tpp1 revealed that the enzyme possesses a robust DNA 3′ phosphatase activity, but an associated 5′ kinase activity was not detected. Tpp1 and Apn1 were identified as the primary constitutive 3′ phosphatase activities in whole-cell extracts, suggesting that these two enzymes, along with Apn2, may represent three alternative pathways for the repair of 3′ phosphate blocking lesions.

In addition to oxidative damage, strand breaks bearing 3′ phosphates are thought to arise in cells as one result of the action of DNA topoisomerases. DNA topoisomerase I (Top1 in yeast) forms a covalent intermediate between the active site tyrosine and the 3′ end of the phosphodiester backbone of DNA. Recently, Nash and colleagues identified a tyrosyl-DNA phosphodiesterase, Tdp1, which cleaves the covalent Top1-DNA phosphotyrosine bond to leave a phosphate at the 3′ terminus (18, 28). It is likely that Tdp1 acts to remove Top1 when transcription or replication complexes cause premature dissociation of the 5′ strand. Consistent with this, *tdp1* mutant yeast is sensitive to the drug camptothecin, which stabilizes the Top1-DNA covalent intermediate (18). This sensitivity is far less than that observed with recombination mutants (16), however, and so alternative pathways for Top1 removal must exist. In the subset of Top1 lesions that are cleaved by Tdp1, 3′ phosphatases must presumably act downstream.

To investigate these possibilities, we examined 3′ phosphate

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TABLE 1. Yeast strains used in this study[a]

Strain	Genotype	Derived from
YW388	*MAT***a** *ade2Δ0 his3Δ-200 leu2Δ-1 met15Δ0 trp1 Δ-63 ura3Δ0*	
YW465	*MATα ade2Δ0 his3Δ-200 leu2Δ-1 met15Δ0 trp1Δ-63 ura3Δ0*	
YW573	*tpp1Δ::MET15*	YW465
YW605	*apn1Δ::HIS3*	YW465
YW619	*tpp1Δ::MET15 apn1Δ::HIS3*	YW605
YW625	*tdp1Δ::URA3*	YW465
YW629	*tpp1Δ::MET15 apn1Δ::HIS3 tdp1Δ::URA3*	YW619
YW652	*rad52Δ::URA3*	YW388
YW653	*TPP1/tpp1Δ::MET15 APN1/apn1Δ::HIS3 RAD52/rad52Δ::URA3*	YW619 × YW652
YW771	*TPP1/tpp1Δ::MET15 APN1/apn1Δ::HIS3 APN2/apn2Δ::KanMX4 RAD52/rad52Δ::URA3*	YW653
YW774	*apn2Δ::KanMX4*	YW771
YW776	*tpp1Δ::MET15 apn2Δ::KanMX4*	YW771
YW778	*tpp1Δ::MET15 apn1Δ::HIS3 apn2Δ::KanMX4*	YW771
YW781	*apn1Δ::HIS3 apn2Δ::KanMX4*	YW771
YW789	*tpp1Δ::MET15 apn1Δ::HIS3 apn2Δ::KanMX4 tdp1Δ::URA3*	YW778

[a] Only alleles that are different from YW388 or YW465 are listed for mutant strains.

removal by using purified enzymes and by evaluating the sensitivity of mutant strains to oxidative and camptothecin-induced DNA damage. We show that Tpp1 is specific and highly efficient in removing phosphates from 3′ termini, in contrast to Apn1. Surprisingly, these studies also revealed that Apn1, but not Tpp1, acts to remove a single nucleotide at a nick in DNA, regardless of the presence of a 3′-terminal lesion. Tpp1 functions synergistically with Apn1 and Apn2 in the repair of lesions induced by treatment with H_2O_2 and bleomycin but not in the repair of lesions induced by treatment with MMS. Furthermore, the loss of Tpp1, Apn1, and Apn2 is lethal in the absence of Rad52-dependent recombinational repair and also sensitizes cells to camptothecin in a *TDP1*-dependent fashion. These findings are discussed in the context of overlapping pathways for the repair of 3′ phosphate blocking lesions.

MATERIALS AND METHODS

Yeast strains. *S. cerevisiae* strains used in this study are listed in Table 1 and are isogenic derivatives of the wild-type strain YW388. Genes were disrupted by using the PCR-mediated one-step replacement technique (4). All disruptions were confirmed by PCR.

Oligonucleotide substrates. The 3′ PG containing substrate was prepared by a two-step oxidation method described by Urata and Akagi (24) as modified by Izumi et al. (9). Briefly, a 22-mer oligonucleotide synthesized with a 3′ glyceryl (Operon) was oxidized at 0°C for 2 h with $NaIO_4$ in 100 mM $NaPO_4$ (pH 6). The oligonucleotide was ethanol precipitated, resuspended in $NaPO_4$, and oxidized in 36% dimethyl sulfoxide (DMSO) with $NaClO_2$ at 25°C for 5 h to afford the 3′ PG. This oligonucleotide, as well as those synthesized with 3′ phosphates (Operon), were 5′ end labeled with [γ-^{32}P]ATP by using 3′ phosphatase-free polynucleotide kinase (Roche Molecular Biochemicals). Oligonucleotides with 3′ hydroxyls were similarly labeled by using T4 polynucleotide kinase (New England Biolabs). Labeled oligonucleotides were then annealed by slow cooling to a twofold molar excess of unlabeled strands. For AP endonuclease assays, a 39-mer oligonucleotide containing a uracil at position 23 was 5′ end labeled with [γ-^{32}P]ATP (8). After being annealed to a complementary strand, the DNA duplex was treated with uracil-DNA glycosylase (New England Biolabs) to generate an abasic site.

Enzyme activity assays. Glutathione *S*-transferase (GST)–Tpp1 and GST-Apn1 were overexpressed and purified from yeast cells as described previously (25). Both enzymes were >95% pure as judged by Coomassie blue staining. To prepare cell extracts of wild-type, *tpp1*, *apn1*, and *tpp1 apn1* cells, overnight cultures grown in YPAD (1% yeast extract, 2% peptone, 2% dextrose, 40 μg of adenine/ml) were diluted to an optical density at 600 nm (OD_{600}) of 0.2 and grown to OD_{600} = 1. Cells were washed with water and lysed with glass beads in buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 M NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 10% glycerol, 2 μg of aprotinin/ml, 1 μg each of leupeptin and pepstatin/ml, and 1 mM phenylmethylsulfonyl fluoride, followed by centrifugation. Crude extracts were diluted with salt-free buffer to a final protein concentration of 0.5 μg/μl. Assays of enzyme activity contained 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 50 μg of bovine serum albumin/ml, 50 fmol of DNA substrate, and either GST-Tpp1, GST-Apn1, or 1 μg of protein from cell extracts in a reaction volume of 10 μl and were incubated at 30°C for 10 min. Reactions were terminated by addition of formamide-EDTA loading buffer and heating to 90°C. Samples were then electrophoresed on denaturing polyacrylamide gels, followed by autoradiography. For rate comparisons, imaging and quantitation was performed by using a PhosphorImager (Molecular Dynamics).

Measurement of drug sensitivity. Sensitivity to H_2O_2 was determined by treating exponentially growing cultures in YPAD with various concentrations of drug at 30°C for 1 h with vigorous shaking. Samples were then serially diluted in water and spread on YPAD plates. Colonies were scored after incubation at 30°C for 2 to 3 days. Fractional survival is calculated for each strain relative to its untreated control. Bleomycin and MMS sensitivities were determined similarly, except that incubation with drug was in synthetic complete medium for bleomycin and in 50 mM KPO_4 (pH 7.5) for 30 min for MMS. Because DNA damage induced by camptothecin occurs during replication (6), camptothecin sensitivity was determined by diluting exponentially growing cultures to 10^4 cells/ml in YPAD containing various concentrations of drug and 2% DMSO. Cultures were shaken at 30°C for 22 h, during which time the untreated wild-type culture was just approaching the end of its exponential growth phase ($OD_{600} < 4$). Cells were serially diluted in water, and CFU were scored as described above. Relative CFU is the number of CFU/ml for a treated culture divided by the CFU/ml for the untreated culture of the same strain.

RESULTS

Tpp1 removes phosphate but not PG 3′ blocking lesions. To understand the cellular role of the Tpp1 phosphatase and gain insight into the drug sensitivity phenotypes described below, it was necessary to first establish its enzymatic specificity. We previously showed that Tpp1 is active on double-stranded but not single-stranded DNA and that it is specific for the 3′ terminus (25). To determine whether Tpp1 can process lesions other than phosphates, three other cleavage activities were examined using purified GST-Tpp1 (25): 3′ PG phosphodiesterase, AP endonuclease, and 3′-5′ exonuclease.

GST-Tpp1 was first incubated with similar duplex DNA substrates containing either a 3′ phosphate or a 3′ PG within a single-nucleotide gap. GST-Tpp1 efficiently removed the phosphate but had no activity against the PG moiety (Fig. 1B, compare lanes 3 and 7). As a positive control, purified GST-Apn1 was similarly incubated and, as expected based on pre-

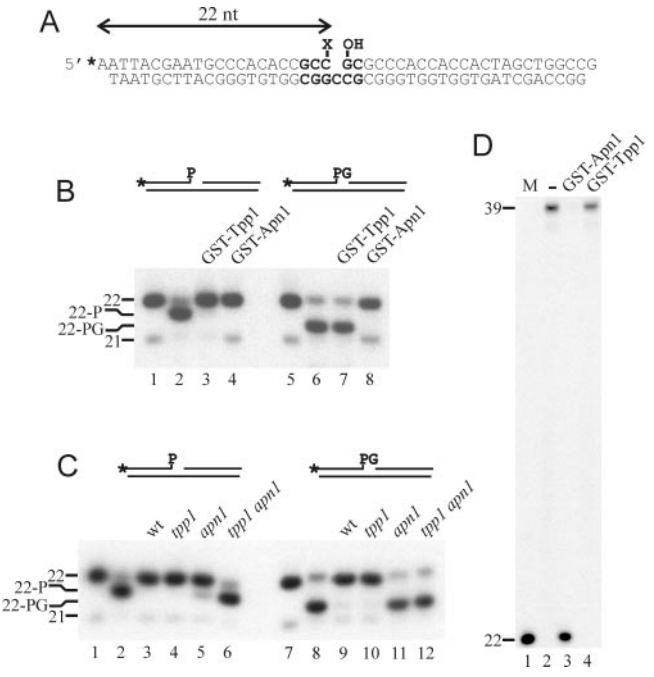


FIG. 1. Tpp1 is specific for removal of 3′ phosphate lesions. (A) 5′-end-labeled DNA substrate used for enzyme assays. X denotes the position of the phosphate (P) or PG. For assays in Fig. 2C, changes were made within the region shown in boldface. (B) The indicated oligonucleotide substrates (50 fmol) were labeled at their 5′ ends and incubated with 10 fmol of GST-Tpp1 or 100 fmol of GST-Apn1 for 10 min at 30°C and electrophoresed on a 7 M urea–18% polyacrylamide gel. (C) Crude cellular extracts (1 μg of protein) from wild-type, *tpp1*, *apn1*, and *tpp1 apn1* strains were similarly incubated as in panel B. Lanes 1 and 2 in panels B and C correspond to the 22-mer oligonucleotide synthesized without and with a 3′ phosphate, respectively. Lanes 5 and 6 in panel B and lanes 7 and 8 in panel C correspond to the 22-mer containing a 3′ hydroxyl or 3′ PG, respectively. Note that phosphate- and PG-terminated oligonucleotides migrate faster in the gel than hydroxyl-terminated oligonucleotides of the same size. (D) GST-Apn1 (100 fmol) or GST-Tpp1 (100 fmol) was incubated with a 39-nucleotide double-stranded oligonucleotide substrate (50 fmol) containing an abasic site at position 23 for 10 min at 30°C and electrophoresed on a 7 M urea–18% polyacrylamide gel. Lane 1 contains a 22-mer marker corresponding to the expected incision product.

vious results (11), catalyzed the removal of both 3′-terminal lesions. We next examined 3′-terminal lesion processing in extracts from wild-type, *tpp1*, *apn1*, and *tpp1 apn1* yeast cells. Similar to our previous findings (25), 3′ phosphatase activity was present in wild-type, *tpp1*, and *apn1* cell extracts and yet was not detected in extracts from *tpp1 apn1* cells, indicating that Tpp1 and Apn1 function as the predominant constitutive pathways for the removal of 3′ phosphates in *S. cerevisiae* (Fig. 1C, lanes 1 to 6). In addition to removing 3′ phosphates, extracts from wild-type and *tpp1* cells, but not *tpp1 apn1* cells, also catalyzed the removal of 3′ PG lesions (Fig. 1C). In contrast, the 3′ PG was not removed by cell extracts lacking only Apn1 (lane 11), demonstrating that, like GST-Tpp1, the native Tpp1 enzyme cannot remove 3′ PGs.

Tpp1 is not an AP endonuclease. It has not yet been examined whether the class of PNK-related 3′ phosphatases might also possess AP endonuclease activity. We generated an abasic site in a double-stranded oligonucleotide by using uracil glycosylase. Unlike GST-Apn1, GST-Tpp1 had no ability to cleave the damaged strand at enzyme concentrations that can completely remove the 3′ phosphate from an equivalent amount of substrate (Fig. 1D).

Apn1 but not Tpp1 is a gap-generating 3′ nuclease. We previously observed that when a nicked oligonucleotide substrate was used in experiments with crude cellular extracts (in contrast to the gapped substrate used above) the 3′-terminal nucleotide was removed, as well as the 3′ phosphate (25). Nucleotide removal was detected with *tpp1* but not with *apn1* strains, suggesting that the nuclease activity might be encoded by *APN1*. Although this finding supported the anticipated lack of Tpp1 nuclease activity, it was surprising based on the previous characterization of Apn1 (12). To explore these predictions in more detail, the reactions of purified GST-Tpp1 and GST-Apn1 with a nicked 3′ phosphate substrate were monitored over time (Fig. 2A). GST-Tpp1 catalyzed a time-dependent removal of only the 3′ phosphate, demonstrating that the enzyme is not an exonuclease. In contrast, GST-Apn1 sequentially removed the phosphate and then the 3′-terminal nucleotide, confirming that the nuclease activity observed in cell extracts was due to Apn1 itself. Consistent with the fact that all other Apn1 cleavage reactions leave a 3′ hydroxyl, the labeled product strand comigrated with a 3′ hydroxyl-terminated 21-mer marker and could be extended by exonuclease-deficient Klenow (Fig. 2B). Interestingly, although removal of the first nucleotide by GST-Apn1 was essentially complete after 5 min, the enzyme did not proceed to remove additional nucleotides even after a further 5 min of incubation (Fig. 2A). This suggested that GST-Apn1 preferentially removes only one nucleotide to leave a single-nucleotide gap.

This possibility was tested by using a panel of DNA substrates containing different modifications at their 3′ and 5′ ends in the context of a nick or a single-nucleotide gap. In addition to removing a nucleotide after first taking off a 3′ phosphate (Fig. 2C, lanes 1 to 4), GST-Apn1 also removed a nucleotide from a substrate containing a 3′ hydroxyl, regardless of the phosphorylation status of the 5′ terminus (Fig. 2C, lanes 9 to 12 and lanes 13 to 16). The enzyme did not remove a nucleotide from the 3′ end of a single-nucleotide gap, however (Fig. 2C, lanes 5 to 8). Likewise, it removed only the phosphate from the 3′ end of a single-nucleotide gap without proceeding to take off the nucleotide, regardless of the position of the gap in the sequence (Fig. 2C, compare lane 2 with lanes 18 and 22). Taken together, these results indicate that, at least in vitro, Apn1 creates a single-nucleotide gap as its terminal product. This product appears in a delayed fashion, however. This is explained by kinetic studies showing that the AP endonuclease activity of Apn1 was comparable to its 3′ phosphatase activity, whereas it was significantly less efficient in removing a nucleotide from the 3′ end (Fig. 3).

Tpp1, Apn1, and Apn2 represent overlapping pathways for repair of oxidative DNA damage. We conclude from the above that Tpp1 specifically removes 3′ phosphates, unlike the promiscuous phosphodiesterase activities of the AP endonucleases that support the removal of many 3′ lesions as well as nucleotides. To examine the role of these Tpp1, Apn1, and Apn2 activities in the repair of 3′ blocking lesions in vivo, we assessed the sensitivity of mutant strains to different DNA-damaging agents. H_2O_2 treatment causes damage to deoxyri-

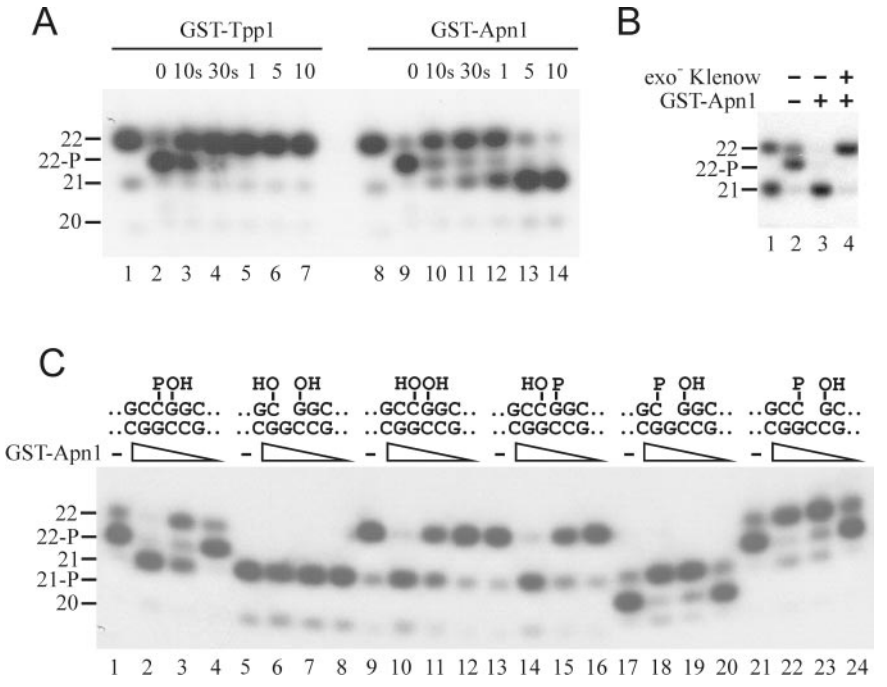


FIG. 2. Apn1 processes strand breaks to single-nucleotide gaps. (A) GST-Tpp1 (10 fmol) or GST-Apn1 (100 fmol) was incubated with a DNA substrate (50 fmol) containing a 3′ phosphate at a nick. At the times indicated, aliquots were withdrawn and quenched by the addition of formamide sample buffer and heating to 90°C. These samples, as well as those in panels B and C, were electrophoresed on 7 M urea–12% polyacrylamide gels. Lanes 1 and 8 contain the corresponding 22-mer oligonucleotide synthesized without a 3′ phosphate. (B) GST-Apn1 (100 fmol) was incubated with the substrate from panel A for 10 min. The reaction was then divided into two tubes and quenched (lane 3), or else 100 μM dCTP and 0.5 U of exo⁻ Klenow added for an additional 5 min at 37°C (lane 4). Lane 1 contains a mixture of the corresponding 21- and 22-nucleotide hydroxyl-terminated markers. (C) GST-Apn1 activity was assayed by incubating the indicated substrates with either no protein (−) or 100, 10, or 1 fmol of GST-Apn1. The configuration surrounding the strand breaks is illustrated. In all, the major product seen with 100 fmol of GST-Apn1 corresponds to a single-nucleotide gap.

bose sugars, resulting in DNA strand breaks bearing 3′-blocking lesions, including 3′ phosphates and 3′ PGs (7). Single mutants of *tpp1*, *apn1*, or *apn2* were no more sensitive to H_2O_2 than wild-type cells, demonstrating a substantial redundancy in oxidative repair pathways (Fig. 4A). In contrast, *tpp1 apn1* and *apn1 apn2* cells demonstrated a similar marked increase in H_2O_2 sensitivity, while the *tpp1 apn1 apn2* triple mutant was exquisitely sensitive, ca. 400-fold more so than either double mutant at 2.5 mM H_2O_2. Interestingly, *tpp1 apn2* cells were only as sensitive as the wild-type strain, suggesting that Apn1 plays a more significant role than Tpp1 or Apn2 in the repair of oxidative DNA damage.

The predominant forms of damage left by bleomycin are strand breaks containing 3′ PGs and abasic sites due to hydrogen abstraction from the C-4′ position of deoxyribose sugars in DNA (reviewed in reference 19). The two lesions are formed in comparable quantities, while the formation of 3′ phosphates, caused by abstraction from C-1′, occurs less often. In contrast to the similar H_2O_2 hypersensitivity demonstrated by *tpp1 apn1* and *apn1 apn2* double mutants, only the latter strain was significantly hypersensitive to bleomycin (Fig. 4B). This result suggests that the 3′ phosphodiesterase and AP endonuclease activities of Apn1 and Apn2 are more important for the repair of the primary forms of damage left by bleomycin. However, similar to treatment with H_2O_2, the *tpp1 apn1 apn2* triple mutant was significantly more sensitive to bleomycin than the *apn1 apn2* strain, indicating that Tpp1 plays an important role in removal of 3′ phosphates in the absence of Apn1 and Apn2.

Importantly, the absence of Tpp1 function did not sensitize cells to all forms of DNA damage. UV radiation induces multiple lesions in DNA including cyclobutane pyrimidine dimers and (6-4) photoproducts, but not strand breaks or 3′ phosphates (7). Consistent with this, loss of *TPP1* alone or in combination with a disruption of *APN1* and/or *APN2* did not hypersensitize cells to UV radiation (data not shown). As an additional control for a DNA repair event that occurs without a known requirement for 3′ phosphatase activity, we also tested strains for sensitivity to MMS. Bases alkylated by MMS are removed by DNA glycosylases generating noncoding abasic sites (7). These sites are cleaved by AP endonucleases, followed by repair synthesis and ligation during BER. Disruption of *APN2* in an *apn1* strain resulted in a large increase in MMS sensitivity (Fig. 4C), a finding consistent with the previously demonstrated overlapping functions of the two enzymes in processing abasic lesions (2, 13). In contrast, disruption of *TPP1* in *apn1*, *apn2*, or *apn1 apn2* strains did not affect sensitivity to the drug. This indicates that Tpp1 is not involved in the repair of abasic sites, a finding consistent with the biochemical data.

Tpp1, Apn1, and Apn2 represent overlapping pathways for repair of Tdp1-generated 3′ phosphates. We hypothesized that the loss of 3′ phosphatase activity in the *tpp1 apn1 apn2* triple

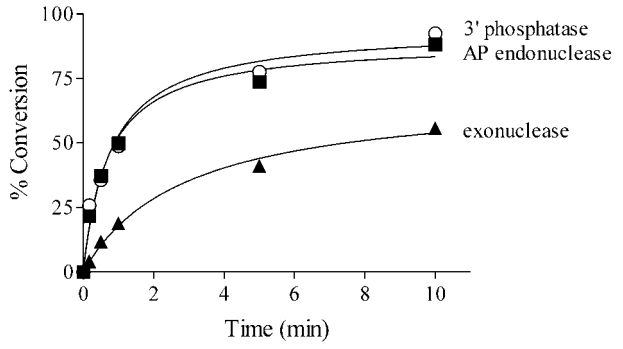


FIG. 3. Kinetic comparison of GST-Apn1 enzymatic activities. Time course of GST-Apn1 activity to determine the relative rate at which the enzyme removed a 3′ phosphate at a single-nucleotide gap (■), a nucleotide at a nick (▲), or a cleaved abasic site (○). All reactions contained 100 fmol of GST-Apn1 and 50 fmol of substrate. Reactions were incubated at 30°C and terminated by using formamide sample buffer and heating to 90°C. Samples were electrophoresed on denaturing polyacrylamide gels, followed by imaging and quantitation with a PhosphorImager. Calculations of the percent conversion were based on the disappearance of substrate.

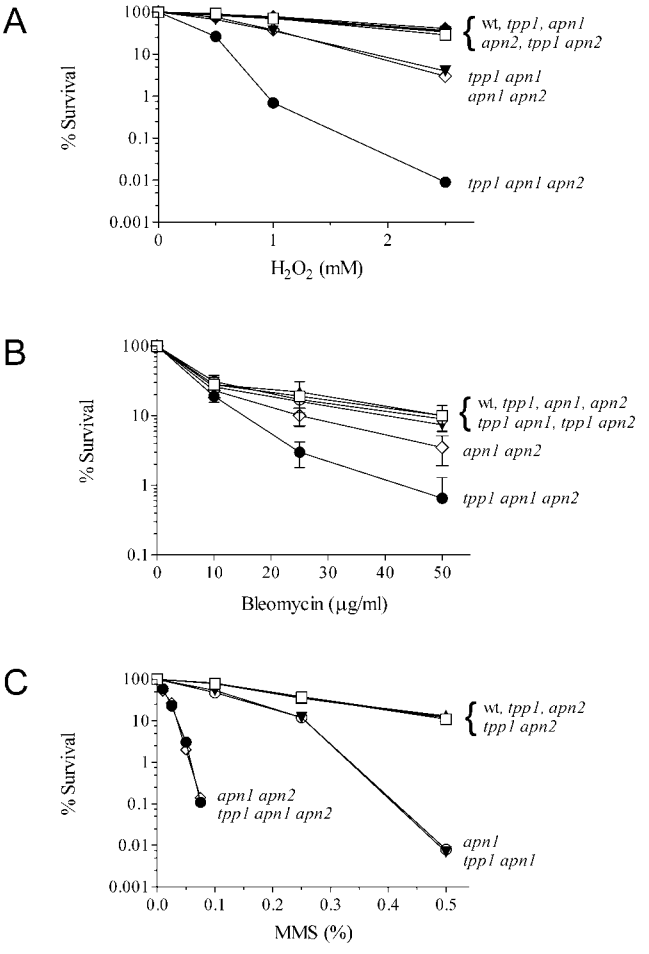


FIG. 4. Different patterns of sensitivity of 3′ phosphatase-deficient strains to H_2O_2, bleomycin, and MMS. Cells were treated with H_2O_2 (A), bleomycin (B), or MMS (C) at the concentrations indicated, and survival was scored relative to the untreated strain as described in Materials and Methods. Curves represent the mean ± the standard deviation of at least two independent experiments for each strain. Strains and symbols are as follows: wild-type, □; *tpp1*, ▲; *apn1*, ○; *apn2*, △; *tpp1 apn1*, ▼; *tpp1 apn2*, ▽; *apn1 apn2*, ◇; *tpp1 apn1 apn2*, ●.

mutant might also render cells hypersensitive to camptothecin to an extent similar to *tdp1* mutants, but only if direct removal of Tdp1-generated 3′ phosphates is required for ultimate resolution of disrupted Top1 complexes by this pathway. Indeed, the *tpp1 apn1 apn2* triple mutant exhibited a marked decrease in cell viability in the presence of camptothecin compared to wild-type, *tpp1*, *apn1*, and *apn2* mutant strains (Fig. 5A). Among the double mutants, only *tpp1 apn1* reproducibly showed an intermediate level of sensitivity in the liquid outgrowth assay. We note, though, that a difference was consistently observed when the wild type was compared with the *apn1 apn2* mutant in assays in which yeast were spotted to plates containing camptothecin (not shown). Surprisingly, the level of sensitivity of phosphatase-deficient strains was in fact substantially greater than that observed for an isogenic *tdp1* strain, which showed only a very slight sensitization at the same camptothecin concentrations (Fig. 5B). Most importantly, deletion of *TDP1* suppressed camptothecin sensitivity of the *tpp1 apn1* and *tpp1 apn1 apn2* mutant strains to the level of the *tdp1* mutant (Fig. 5B). This confirms that the majority of camptothecin-induced 3′-blocking lesions processed by the combined action of Tpp1, Apn1, and Apn2 are generated by Tdp1 and therefore that Tdp1 acts upstream of Tpp1, Apn1, and Apn2 in the same Top1 repair pathway. These findings further indicate that the alternative pathway that repairs Top1 complexes in the absence of Tdp1 is largely unable to act when it is the Tdp1-generated 3′ phosphate that persists.

DNA 3′ phosphatase activity is required for cell survival in the absence of *RAD52*. Previous experiments by Nitiss and Wang have demonstrated the importance of recombinational repair to the resolution of Top1-mediated DNA damage as judged by the marked camptothecin sensitivity of *rad52* mutants (16). Similarly, recent studies have indicated that multiple repair pathways, including recombination, are elicited in response to oxidative DNA damage (21). To determine if the recombinational repair pathway overlaps with Tpp1, Apn1, and Apn2-dependent processing of strand breaks, we disrupted *RAD52* in a diploid strain heterozygous for *tpp1*, *apn1*, and *apn2*. After tetrad dissection, the quadruple *tpp1 apn1 apn2 rad52* haploid mutant was inviable, and both *tpp1 apn1 rad52* and *apn1 apn2 rad52* triple mutants exhibited extremely poor growth, growing much more slowly than either the *apn1 rad52* mutant or the *rad52* mutant alone (Fig. 6).

DISCUSSION

DNA strand breaks containing 3′ lesions occur frequently in cells. These lesions represent a block to repair synthesis and ligation and therefore pose a significant threat to genomic stability. We have found that in *S. cerevisiae* the repair of 3′ blocking lesions involves at least three distinct enzymes that display significant, but not complete, redundancy. Two enzymes, Apn1 and Apn2, have similar broad substrate specific-

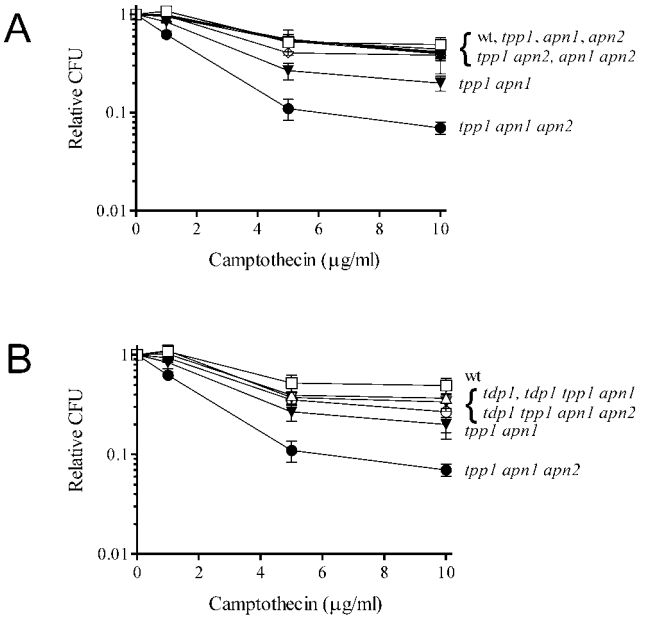


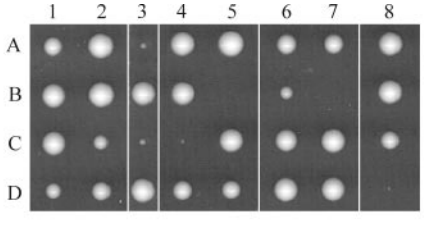
FIG. 5. Loss of *TDP1* suppresses camptothecin sensitivity of 3′ phosphatase-deficient strains. Cells were grown in the presence of the indicated concentrations of camptothecin for 22 h, and CFU were scored relative to the untreated strain. Curves represent the mean ± the standard deviation of three independent experiments for each strain. (A) Comparison of *tpp1*, *apn1*, and *apn2* single and multiple mutants. (B) Suppression of *tpp1 apn1* and *tpp1 apn1 apn2* camptothecin sensitivity by mutation of *TDP1*. Strains and symbols are as follows: wild-type, □; *tpp1*, ▲; *apn1*, ■; *apn2*, ◆; *tpp1 apn1*, ▼; *tpp1 apn2*, +; *apn1 apn2*, ◇; *tpp1 apn1 apn2*, ●; *tdp1*, ◇; *tdp1 tpp1 apn1*, ▽; *tdp1 tpp1 apn1 apn2*, ○.

ities. The other, Tpp1, is highly efficient and specific for a single lesion type, the 3′ phosphate.

Specific repair of 3′ phosphate lesions by Tpp1. The phosphate specificity of Tpp1 was initially suggested by alignments of putative polynucleotide kinase DNA 3′ phosphatases that showed the uniform presence of motifs characteristic of the "DDDD" superfamily of phosphohydrolases (10, 14, 22, 25). Members of this superfamily include nonspecific acid phosphatases, PG phosphatases, histidinol phosphatases, phosphoserine phosphatases, and others. Their strong structural conservation suggested a common mode of recognition and removal of phosphates in various substrate contexts. However, the DDDD superfamily is itself a member of an even larger superfamily of hydrolases, typified by L-2-haloacid dehydrogenase, that cleave a much greater variety of substrates (1). It was thus possible that Tpp1 was more promiscuous than initially recognized. We have shown that Tpp1, either purified as a GST fusion protein or in its native form in cellular extracts, is highly efficient in removing 3′ phosphates but does not remove 3′ PGs or terminal nucleotides or cleave abasic sites. Thus, unlike the 3′ phosphodiesterase and nuclease activities of Apn1 and Apn2 (11, 23), Tpp1 appears to exhibit only a robust 3′ phosphatase activity, a finding critical to the interpretation of DNA damage sensitivities of mutant strains.

While this study was in preparation, Betti et al. described a preliminary characterization of the enzyme ZmDP2 from *Zea mays* (3), which bears significant structural homology to Tpp1 and similarly lacks a 5′ kinase domain (3, 25). They reported that ZmDP2 has a 3′ phosphodiesterase activity in addition to being a 3′ phosphatase, although this activity was extremely weak. In our studies, GST-Apn1 could quantitatively convert the 3′ PG to a hydroxyl, but we saw no detectable conversion at GST-Tpp1 concentrations in substantial excess over those required to quantitatively convert 3′ phosphates (Fig. 1 and data not shown). Further work with more active preparations of ZmDP2 should clarify whether this represents a true difference between the yeast and plant 3′ phosphatases.

Controlled excision of single nucleotides at nicks by Apn1. In the course of examining the substrate specificity of Tpp1, we observed that Apn1 appears to recognize different types of DNA damage, including strand breaks with or without 3′-blocking groups and abasic sites, and processes them similarly to create a common final product, a single-nucleotide gap. In the case of nicks, it is likely that the corresponding Apn1 nuclease activity was not previously recognized in standard radiometric exonuclease assays due to its self-limited nature (12). Indeed, this activity might be seen as either a 3′-5′ exonuclease or, perhaps more correctly, as a nick-directed endonuclease. Our interpretation is that Apn1 must make contacts with the nucleotide on the 3′ side of the nick prior to removing the nucleotide on the 5′ side of the nick. The enzyme in effect uses the 3′ nucleotide as a reference from which to measure so that at most a single-nucleotide gap is generated. It is intriguing that even though gaps of two nucleotides are not generated by Apn1, they are able to act as substrate for its phosphatase reaction (not shown). It will be of interest to explore the structural basis for these apparently different substrate requirements. In the nuclease reaction, the nucleotide reference on the 3′ side of the nick is used the same whether its 5′



Genotype	Position	Genotype	Position
wild-type	D7	*apn1 apn2*	A4
tpp1	A5, D3	*apn1 rad52*	B6, C2, D1
apn1	A8, C5, C7	*apn2 rad52*	D2, D5
apn2	B3, C1, D6	*tpp1 apn1 apn2*	C6
rad52	D4	*tpp1 apn1 rad52*	C3, C4
tpp1 apn1	A2, B1	*tpp1 apn2 rad52*	A1, A7
tpp1 apn2	B2, B4, B8	*apn1 apn2 rad52*	A3, D8
tpp1 rad52	A6, C8	*tpp1 apn1 apn2 rad52*	B5, B7

FIG. 6. Synthetic lethality of *tpp1 apn1 apn2* with *rad52*. The diploid strain YW771 (*TPP1/tpp1Δ::MET15 APN1/apn1Δ::HIS3 APN2/apn2Δ::KanMX4 RAD52/rad52Δ::URA3)* was sporulated and dissected. Spore genotypes were determined by replica plating and are indicated in the table below. Genotypes of inviable spores were inferred from the segregation pattern. The tetrads shown are representative of the growth phenotypes consistently observed across the >50 tetrads analyzed and were additionally confirmed by restreaking to single colonies (not shown).

position contains a phosphate or a hydroxyl. Further, a 3′ lesion is not required because 3′ OH-terminated nicks are sufficient to act as substrates in this reaction. Such results obtained using purified proteins must be interpreted with caution, however, and may not reflect the actual reactions performed by the multiprotein repair complexes within cells. We suggest that in the cell simple nicks are likely to be rapidly ligated, preventing the relatively slow nucleotide excision step by Apn1. However, when ligation occurs inefficiently, for example, with a mismatched 3′ base, Apn1 might compete with ligase and remove the nucleotide. Further experiments are required to explore this possibility.

Repair of oxidative damage by Tpp1, Apn1, and Apn2. Apn1 and Tpp1 are the primary 3′ phosphatase activities present in crude extracts of *S. cerevisiae* (25) (Fig. 1C). The absence of Apn2 does not indicate it has no role in repair, however, since it is known to be induced after DNA damage (2). Indeed, Apn1 and Apn2 have previously been shown to function as alternative pathways for the repair of abasic sites, as reflected by the synergistic increase in sensitivity of *apn1 apn2* cells treated with MMS (2, 13). A similar functional overlap of Tpp1 with Apn1 and/or Apn2 was not observed in response to MMS, however, indicating that Tpp1 is not required for the repair of abasic lesions.

Unk et al. recently found that *apn1 apn2* cells showed an increased sensitivity to oxidative damage induced by H_2O_2 after the introduction of a catalytically inactive mutant of Apn2 (23). The increased sensitivity was suggested to result from unproductive binding of mutant Apn2 to 3′-terminal lesions, preventing their removal by an alternative repair pathway. Our finding that *tpp1 apn1 apn2* cells were markedly more sensitive to H_2O_2 than *apn1 apn2* cells strongly implicates Tpp1 as this third pathway of repair. Indeed, the extreme sensitivity of the *tpp1 apn1 apn2* triple mutant makes it unlikely that another 3′ phosphatase exists in *S. cerevisiae*. Moreover, considering the extent of the *tpp1* mutant effect in light of the lesion specificity of Tpp1 suggests that most oxidative strand breaks bear 3′ phosphates.

The *tpp1 apn1 apn2* triple mutant was also significantly more sensitive than either double mutant to bleomycin. However, unlike the situation with H_2O_2, *apn1 apn2* cells but not *tpp1 apn1* cells were found to be hypersensitive to the drug. This latter pattern of sensitivity would be predicted if the primary lesions generated by bleomycin are, in fact, 3′ PGs and abasic sites rather than 3′ phosphates since Tpp1 is only active on the latter lesion. This would not predict the large increase in bleomycin sensitivity observed in the *tpp1 apn1 apn2* triple mutant, however. It is not clear at present if this hypersensitivity instead reflects a generation of 3′ phosphates as a secondary event in the face of delayed repair of bleomycin damage, perhaps by decomposition of the primary bleomycin lesions into 3′ phosphates, or even by generation of new lesions at previously undamaged sites.

Pathways for repair of Top1-DNA covalent complexes. Ordinarily the strand break generated by Top1 is sealed by a simple reversal of its catalytic mechanism. When this is not possible, due to the presence of camptothecin or premature dissociation of the 5′ strand, it is clear that the covalently bound protein must be removed during repair of the break. The tyrosyl-DNA phosphodiesterase Tdp1 is a primary candidate, but attempts to verify its role have been complicated by the fact that active alternative mechanisms exist that greatly minimize the sensitivity of *tdp1* mutants to camptothecin (18). Our finding that *tdp1* suppresses the hypersensitivity of *tpp1 apn1 apn2* mutants to camptothecin strongly suggests that the 3′ phosphate lesion left by Tdp1 cannot be processed efficiently by these alternative mechanisms. This provides the strongest evidence to date that Tdp1 is in fact substantially active in the repair of Top1-DNA complexes and establishes the sequential action of Tdp1 followed by 3′ phosphatase as a major pathway. It will be of great interest to explore in more detail the interplay between this and the unknown alternative pathway(s) of repair.

Overlapping pathways for the repair of endogenously generated DNA damage. The finding that *tpp1 apn1 apn2* cells grow similarly to the wild type but die when *RAD52* is deleted suggests that recombination might serve as an alternative mechanism for dealing with endogenously generated DNA lesions that are normally repaired by the combined action of Tpp1, Apn1, and Apn2. Identifying the specific nature of these lesions is complicated by the fact that this group of enzymes has multiple activities that might each be required for full viability. Apn1 and Apn2 account for the majority of AP endonuclease activity in yeast (11, 13, 20), and so abasic sites created by frequent spontaneous base loss (27) likely contribute by causing replication fork stalling. Such forks require Rad52-dependent restart (5). This does not account for the fact that mutations of *tpp1* and *apn2* have equivalent detrimental effects on the growth of *rad52* mutants, however, because Tpp1 is not an AP endonuclease. Indeed, our biochemical data indicate that at least a portion of the lesions leading to cell death must be strand breaks with 3′ phosphates. In the absence of Tpp1, Apn1, and Apn2, each of which contributes to 3′ end processing, such lesions would persist and lead to replication fork collapse in which the single-strand break is converted into a double-strand break. In this way, persistent 3′ blocking lesions could potentially be removed by one of the pathways known to trim 3′ ends during recombination, most notably the Rad1-Rad10 nuclease (17). That these lesions might be generated by endogenous ROS is suggested by the similarity in the observed genetic patterns of H_2O_2 sensitivity and slow growth in the *rad52* background, as well as by the known large burden of oxidative damage suffered in metabolizing cells (15). Finally, an alternative explanation for the synthetic lethality of *tpp1 apn1 apn2 rad52* mutants would be that loss of *RAD52* leads, by an unknown mechanism, to an increased production of 3′ phosphate lesions. Further work is required to understand which of these alternatives is correct.

In summary, the results presented here establish that Tpp1 participates in the repair of DNA strand breaks caused by oxidation, bleomycin, Top1, and endogenous sources. Consistent with these findings, Whitehouse et al. have recently provided evidence for the involvement of hPNKP in single-strand break repair (26). It is unclear, however, why three redundant pathways have evolved to repair 3′ phosphate lesions, since the multifunctional AP endonucleases appear well suited for both constitutive and inducible removal of 3′ phosphates during BER. Perhaps multiple enzyme activities are required to process the range of lesions induced by ROS, in which case Tpp1 could act to remove 3′ phosphates whereas Apn1 and Apn2

function primarily in the repair of abasic and nonphosphate lesions. The apparently high percentage of 3′ phosphate lesions induced by oxidation is consistent with the need for an efficient and specific 3′ phosphatase. Alternatively, the high specificity and efficiency of Tpp1 may indicate that the enzyme evolved for an as-yet-unidentified cellular task that involves only 3′ phosphates.

ACKNOWLEDGMENTS

This work was supported in part by the Pew Scholars Program in the Biomedical Sciences of the Pew Charitable Trusts and Public Health Service grant CA-90911 (T.E.W.) and training grant 5T32HL07157 (J.R.V).

We thank Tadahide Izumi (University of Texas Medical Branch) for technical information regarding the preparation of the 3′-PG-containing oligonucleotide.

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