Relationships between yeast Rad27 and Apn1 in response to apurinic/apyrimidinic (AP) sites in DNA

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ABSTRACT

Yeast Rad27 is a 5′→3′ exonuclease and a flap endonuclease. Apn1 is the major apurinic/apyrimidinic (AP) endonuclease in yeast. The rad27 deletion mutants are highly sensitive to methylmethane sulfonate (MMS). By examining the role of Rad27 in different modes of DNA excision repair, we wish to understand why the cytotoxic effect of MMS is dramatically enhanced in the absence of Rad27. Base excision repair (BER) of uracil-containing DNA was deficient in rad27 mutant extracts in that (i) the Apn1 activity was reduced, and (ii) after DNA incision by Apn1, hydrolysis of 1–5 nucleotides 3′ to the baseless sugar phosphate was deficient. Thus, some AP sites may lead to unprocessed DNA strand breaks in rad27 mutant cells. The severe MMS sensitivity of rad27 mutants is not caused by a reduction of the Apn1 activity. Surprisingly, we found that Apn1 endonuclease sensitizes rad27 mutant cells to MMS. Deleting the APN1 gene largely restored the resistance of rad27 mutants to MMS. These results suggest that unprocessed DNA strand breaks at AP sites are mainly responsible for the MMS sensitivity of rad27 mutants. In contrast, nucleotide excision repair and BER of oxidative damage were not affected in rad27 mutant extracts, indicating that Rad27 is specifically required for BER of AP sites in DNA.

INTRODUCTION

Excision repair of DNA functions to remove DNA lesions, thus suppressing mutagenesis and carcinogenesis induced by DNA damaging agents. Depending on whether the modified base is removed as a free base or an oligonucleotide, excision repair is divided into two major mechanisms: base excision repair (BER) and nucleotide excision repair (NER), respectively. BER is a major repair mechanism for endogenous DNA damage such as uracil residues in DNA, apurinic/apyrimidinic (AP) sites and oxidative damage. NER is a versatile repair pathway in that it is capable of removing a variety of DNA lesions such as those induced by UV, cisplatin, acetylaminofluorene (AAF) and polycyclic aromatic hydrocarbons (1–3).

The NER pathway can be conceptually divided into five biochemical steps: damage recognition, incision, excision, repair synthesis and DNA ligation. Over 20 NER proteins have been identified and in vitro reconstitution from purified components has been achieved for transcription-independent NER (global genome repair) (4–6). BER is initiated with direct base removal by a specific DNA glycosylase. Two classes of DNA glycosylases have been identified: glycosylases with or without associated AP lyase activities. The downstream repair events are dictated by the initiating glycosylase. After excision by a glycosylase without associated AP lyase (e.g., repair of a uracil residue in DNA), an AP site is formed in DNA. The AP site is incised at its 5′ side by an endonuclease, generating a 3′-hydroxyl group and a 5′-deoxyribose phosphate moiety. In yeast, this reaction is catalyzed by the Apn1 endonuclease (7). After removal of the 5′-deoxyribose phosphate moiety and DNA repair synthesis, BER completes with a DNA ligation step (7–10). AP sites formed as a result of depurination or depyrimidination can also be repaired by this BER pathway. If BER is initiated by a glycosylase with associated AP lyase (e.g., repair of a thymine glycol residue in DNA), the resulting AP site is cleaved at its 3′ side by the AP lyase activity immediately after base excision (11–16). Thus, base excision and AP site cleavage reactions are conceptually mediated by a single protein. The 3′ baseless sugar phosphate at the DNA nick is subsequently removed, and the repair is completed by sequential DNA repair synthesis and ligation.

In yeast, NER requires the nuclelease activity of Rad2 protein (17,18). Another member of the Rad2 nuclease family is Rad27 (Rlh1) (19–21). Rad27 possesses a 5′→3′ exonuclease activity and a flap endonuclease activity (21,22). Inactivation of Rad27 results in a temperature-sensitive phenotype for growth, a strong mutator phenotype, chromosome instability and a severe sensitivity to methylmethane sulfonate (MMS) (19,20,23). Apparently, Rad27 protein plays multiple roles in cells: processing Okazaki fragments during DNA replication (24–28), maintaining the stability of DNA sequences flanked by short direct repeats (29) and stabilizing micro- and minisatellite DNA sequences (30). The MMS sensitivity suggests that Rad27 may play a role in DNA repair. However, the precise role of Rad27 in yeast DNA repair is not known. Recently, it was shown that FEN1, the Rad27 homolog in higher eukaryotes, is involved in the repair of oxidized AP sites in human cell extracts (31) and a synthetic AP site analog (3-hydroxy-2-hydroxymethyltetrahydrofuran) in a Xenopus laevis in vitro BER system (32).

Rad27 is required to maintain cellular resistance to MMS, but not ionizing radiation, and only plays a minor role against

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UV-induced cytotoxicity (19). The mechanism responsible for this apparent specificity is unknown. By studying the role of Rad27 in different modes of DNA excision repair, we wish to gain insights into the cellular function of Rad27 in response to various DNA damaging agents. In this report, we (i) demonstrate that Rad27 is specifically required for yeast BER of AP sites in DNA; (ii) show that Apl1 endonuclease sensitizes rad27 mutant cells to MMS; and (iii) offer a molecular mechanism for the differential response of rad27 mutant cells to various DNA damaging agents.

**MATERIALS AND METHODS**

**Strains**

The *Saccharomyces cerevisiae* wild-type strains used were SX46A (33) and JJ567 (34). The *apn1* deletion mutants were DRY370 (35) and SX46Aapn1Δ (MATa *apn1::URA3 ade2 his3-532 trp1-289 ura3-52). The *rad10* and *rad27* deletion mutants were BJ2168rad10Δ (36) and SX46Arad27Δ (19), respectively.

**Preparation of DNA substrates for *in vitro* repair**

To prepare AAF-modified DNA, plasmid pUC18 (100 µg) was treated with 3 µM *N*-acetoxy-2-acetylaminofluorene (activated form of AAF) in the dark for 3 h in 1 ml TE buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA) containing 20% ethanol. Damaged DNA was then purified by centrifugation in a linear 5–20% sucrose gradient to remove nicked plasmid DNA as previously described (37). This treatment yielded approximately five damaged bases per pUC18 molecule on average (37).

Osmium tetroxide-damaged DNA was obtained by incubating the agent (300 µg/ml) with plasmid pUC18 (100 µg) in 300 µl of TES buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, and 100 mM NaCl) at 70°C for 90 min. Damaged DNA was then purified by centrifugation in a linear 5–20% sucrose gradient to remove nicked plasmid DNA as previously described (37). This treatment yielded approximately five damaged bases per pUC18 DNA molecule as determined by sensitive sites to *Escherichia coli* endonuclease III (37).

To prepare uracil-containing DNA, the plasmid was propagated in *E.coli* C326 (ung`. *dut`). Plasmid DNA was then isolated and purified by alkaline lysis and CsCl–ethidium bromide equilibrium centrifugation. The resulting plasmid DNA contained randomly distributed uracil residues in place of some thymine residues.

Single-stranded oligonucleotide (30mer) containing a uracil residue at position 13 and its complementary strand were synthesized in a DNA synthesizer and purified by HPLC (Operon, Inc.). The two oligonucleotide strands were then annealed to form a duplex by incubating equal molar amounts of both oligonucleotides for 5 min at 85°C in TES buffer followed by slow cooling to room temperature. The sequence of the uracil-containing oligonucleotide is 5′-GGATGGGATGCAATT- AACCGGAGGCCGCGGC-3′.

**In vitro NER**

Yeast whole cell extracts for *in vitro* NER were prepared as previously described (38). Standard NER reaction mixture (50 µl) contained 200 ng each of AAF-pUC18 DNA and undamaged pGEM3zf DNA, 45 mM HEPES–KOH (pH 7.8), 7.4 mM MgCl₂, 0.9 mM dithiothreitol, 0.4 mM EDTA, 2 mM ATP, 20 µM each dATP, dGTP and dTTP, 4 µM dCTP, 1 µCi of [γ-32P]dCTP (3000 Ci/mmol), 40 mM phosphocreatine (disodium salt), 2.5 µg of creatine phosphokinase, 4% glycerol, 100 µg/ml bovine serum albumin, 5% polyethylene glycol 8000 and 300 µg of yeast cell-free extracts. After incubation at 26°C for 2 h, plasmid DNA was purified, linearized with HindIII restriction endonuclease, separated by 1% agarose gel electrophoresis and autoradiographed as described previously (37,38). To examine the DNA ligation step of NER, repaired plasmid DNA was purified and loaded directly onto a 1% agarose gel without prior linearization with HindIII. Electrophoresis was performed in the presence of 0.5% ethidium bromide.

**In vitro BER**

The same extracts used for *in vitro* NER were also used for *in vitro* BER and were prepared as previously described (36,37). Standard repair synthesis assays (50 µl each) contained 2 pmol of the 30mer duplex DNA, 45 mM HEPES–KOH (pH 7.8), 7.4 mM MgCl₂, 0.9 mM dithiothreitol, 0.4 mM EDTA, 2 mM ATP, 20 µM each dATP, dGTP and dCtP, 4 µM dTTP, 1 µCi of [α-32P]dTTP (3000 Ci/mmol), 40 mM phosphocreatine (disodium salt), 2.5 µg of creatine phosphokinase, 4% glycerol, 100 µg/ml bovine serum albumin and 50–80 µg of yeast whole cell extracts. After incubation at 23°C for 2 h, DNA was extracted by phenol/chloroform and precipitated in ethanol. Repair products were separated by electrophoresis on a 20% denaturing polyacrylamide gel. Repair synthesis was visualized by autoradiography of the wet gel. For BER assays of plasmid DNA, the reaction buffer was identical to that of the 30mer oligonucleotide repair. After incubation at 30°C for 2 h in 80 µg of yeast extracts, DNA purification, treatments and product detection were identically performed as in NER assays described above.

**Incision assays in yeast extracts**

Prior to incision assays, the uracil-containing strand of the oligonucleotide duplex DNA was labeled at its 5′ or 3′ end with 32P. For 5′ labeling, the 30mer oligonucleotide was labeled by [γ-32P]ATP with T4 polynucleotide kinase, followed by annealing to its complementary strand as described above. For 3′ labeling, a 31mer oligonucleotide was synthesized which was complementary to the uracil-containing strand but with an extra A at its 5′ end. After annealing the two oligonucleotides, a [32P]dTMP was added to the 3′ of the uracil-containing strand by the Klenow fragment of *E.coli* DNA polymerase I. The reaction mixture (20 µl) contained 50 mM Tris–HCl pH 8.0, 10 mM MgCl₂, 1 mM dTTP, 75 µCi of [α-32P]dTTP (3000 Ci/mmol), 10 pmol duplex DNA and 3 U Klenow fragment of *E.coli* DNA polymerase I. After incubation at 30°C for 30 min, DNA was extracted by phenol/chloroform and purified by a Push column (Stratagene, Inc.).

Standard incision assays were performed in a 50 µl reaction mixture containing the [32P]-labeled duplex DNA (0.5 pmol), 45 mM HEPES–KOH (pH 7.8), 7.4 mM MgCl₂, 0.9 mM dithiothreitol, 0.4 mM EDTA, 40 mM phosphocreatine (disodium salt), 4% glycerol, 100 µg/ml bovine serum albumin and 50–80 µg of yeast extracts. After incision reactions at 23°C for various times as indicated, a 5 µl aliquot was used for analysis by electrophoresis on a 20% denaturing polyacrylamide gel. Incision products were detected by autoradiography of the wet gel.
Sensitivity to MMS

Yeast cells grown at 30°C to stationary phase in minimum media were harvested by centrifugation, washed with water and resuspended in 100 mM potassium phosphate (pH 7.0) (4 OD₆0₀ cells/ml). Cells were then treated with various doses of MMS as indicated at 30°C for 2h in 80 µg of the extracts and the repair products were separated by electrophoresis on a 1% agarose gel. Reduction of the linear U-pUC18 DNA (2.7 kb) on ethidium bromide-stained gel resulted from extensive DNA fragmentation initiated by uracil-DNA glycosylase in the extracts. +Uracil, modified DNA; –Uracil, unmodified DNA. Top, ethidium bromide-stained gel; bottom, autoradiogram. (B) BER of the 30mer duplex DNA containing a uracil residue at position 13. In vitro repair was at 23°C for 2h in 50 µg of the extracts and the repair products were separated by electrophoresis on a 20% denaturing polyacrylamide gel. Size markers in nucleotides are indicated on the right.

RESULTS

BER of uracil-containing DNA is deficient in rad27 mutant extracts

BER of uracil residues in DNA is initiated by uracil-DNA glycosylase without associated AP lyase (7,12). In this mode of BER, AP sites are formed as repair intermediates following the glycosylase action. Subsequently, DNA incision 3′ to the AP site requires another enzyme, Apn1 endonuclease in yeast (7). Thus, this pathway is also a major mechanism for the repair of spontaneous or induced AP sites in DNA. To examine the role of Rad27 in this mode of excision repair, we performed in vitro repair in yeast cell-free extracts using plasmid pUC18 DNA containing uracil residues in place of some thymine residues.

Under the conditions used, repair of uracil-containing DNA in yeast cell extracts is specifically mediated by the BER pathway and exclusively initiated by uracil-DNA glycosylase (7). As previously observed (39), incubation of uracil-containing pUC18 in wild-type yeast extracts led to DNA fragmentation due to extensive DNA strand breaks after sequential uracil removal and AP site cleavage (Fig. 1A, lane 1, top panel). Identical incubation with unmodified pUC18 did not result in DNA fragmentation (39). Nevertheless, repair synthesis as monitored by 32P-labeling of the repair patch was readily detected in the remaining full length pUC18 molecules and the smeared pUC18 DNA fragments (Fig. 1A, lane 1). In contrast, such repair synthesis in rad27 mutant extracts was deficient (Fig. 1A, lane 2). To confirm this result, a 30mer duplex DNA containing a site-specific uracil residue at position 13 was used for repair in rad27 mutant extracts. Repair synthesis of this duplex substrate was also deficient (Fig. 1B, compare lanes 1 and 2).

Apn1 activity is reduced in rad27 mutant extracts

Fragmentation of uracil-containing DNA in yeast cell extracts is initiated by uracil-DNA glycosylase. Such DNA fragmentation was also observed in rad27 mutant extracts (Fig. 1A, lane 2, top panel). Thus, uracil-DNA glycosylase was functional in rad27 mutant extracts. To identify the biochemical step(s) affected by Rad27, we examined the second step of the BER pathway: DNA incision.

The site-specific uracil-containing DNA was labeled with 32P at the 5′ end with 32P-labeling of the repair patch was readily detected in the remaining full length pUC18 molecules and the smeared pUC18 DNA fragments (Fig. 1A, lane 1). In contrast, such repair synthesis in rad27 mutant extracts was deficient (Fig. 1A, lane 2). To confirm this result, a 30mer duplex DNA containing a site-specific uracil residue at position 13 was used for repair in rad27 mutant extracts. Repair synthesis of this duplex substrate was also deficient (Fig. 1B, compare lanes 1 and 2).
by the Apn1 endonuclease, generating a labeled 12mer DNA fragment (7). Incision of the AP site by an AP lyase generates a labeled DNA fragment containing a baseless sugar phosphate moiety at its 3′ end, which migrated slower than the 12mer DNA on the denaturing polyacrylamide gel (7). As previously observed (7), 5′ incision at the AP site in wild-type extracts was efficient, incising the majority of input substrate DNA in 10 min (Fig. 2, lanes 1–3). In contrast, 5′ incision in rad27 mutant extracts was deficient and only a minor 5′ incision activity was detected after 30 min incubation (Fig. 2, lanes 4–6). Instead, a small fraction of the DNA was incised 3′ to the AP site (Fig. 2, lanes 4–6). Although the 5′ incision activity was much lower than the 3′ incision (Fig. 2, lanes 4–6), we consistently observed residual 5′ incision activity in rad27 mutant extracts. In comparison, the 5′ incision in apn1 mutant extracts was not detectable and the 3′ incision activity was as weak as in the rad27 mutant extracts (Fig. 4, lanes 7–9).

In yeast, APN1 and RAD27 are two adjacent genes on chromosome XI. Deleting the RAD27 gene did not lead to fortuitous inactivation of the APN1 gene in this rad27 strain as indicated by Reagan et al. (19). Furthermore, we detected APN1 transcripts in rad27 mutant cells by RT–PCR (data not shown). These results indicate that the Apn1 activity is reduced in rad27 mutant extracts, thus leading to deficient repair synthesis of uracil BER (Fig. 1). DNA fragmentation of uracil-containing plasmid in rad27 mutant extracts (Fig. 1A, lane 2) presumably resulted from 3′ AP site incisions, as observed in Figure 2 (lanes 4–6), in addition to the residual 5′ incisions by Apn1.

Apn1 endonuclease sensitizes rad27 mutant cells to MMS

To determine whether the MMS sensitivity of rad27 could be accounted for by deficient Apn1 activity, we quantitatively compared the sensitivity of rad27 with that of apn1 mutants. As reported by Reagan et al. (19), rad27 mutant cells were highly sensitive to MMS (Fig. 3). In contrast to the earlier report by Ramota et al. (35), we observed that apn1 mutant cells were only slightly sensitive to MMS (Fig. 3). This result was reproducible with both our own apn1 strain and the DRY370 strain of Ramota et al. (35). Hence, the dramatic MMS sensitivity of rad27 mutants is not caused by a reduction of the Apn1 activity. To confirm this conclusion, we transformed rad27 mutant cells with the APN1 gene on a plasmid under the control of the GAL1 promoter. Surprisingly, overexpression of Apn1 in rad27 mutant cells resulted in a hypersensitivity to MMS (Fig. 3). These observations led us to suspect that MMS sensitivity of rad27 may be largely caused by the residual Apn1 activity in the mutant cells. To test this notion, we disrupted the APN1 gene of the rad27 mutant and measured its MMS sensitivity. As shown in Figure 3, completely inactivating the Apn1 activity drastically enhanced the resistance of rad27 mutant cells to MMS. The rad27 apn1 double mutant was only slightly sensitive to MMS, close to the apn1 single mutant sensitivity (Fig. 3).

Inactivating the APN1 gene, however, did not suppress the temperature-sensitive growth of rad27 mutant cells at 37°C (data not shown), suggesting that the molecular bases for MMS sensitivity and temperature-sensitive growth phenotypes are different. These results demonstrate that Apn1 endonuclease sensitizes rad27 mutant cells to the killing effect of MMS.

Incision by AP endonuclease generates a DNA strand break with a 3′ hydroxyl group and a 5′ deoxyribose phosphate moiety (40). Yeast DNA polymerase β does not play a significant role in BER (41,42; X.Wu and Z.Wang, unpublished results), thus excluding the possibility of removing the 5′ deoxyribose phosphate by the dRPase activity of the polymerase. Based on studies of the FEN1 protein (a Rad27 homolog) in higher eukaryotes (31,32,43), we suspected that the 5′ deoxyribose phosphate moiety may be removed by Rad27 in yeast through its nuclease activity. To test this possibility, we examined the repair step after DNA incision. We first labeled the site-specific uracil-containing DNA at its 3′ end by enzymatic addition of [32P]dTMP. The DNA was treated with E.coli uracil-DNA glycosylase and endonuclease IV, an Apn1 homolog (44), to generate a DNA fragment (Fig. 4A). As previously observed (7), the DNA was treated with E.coli uracil-DNA glycosylase (1 U) and endonuclease IV (2.5 ng) at 30°C for 30 min to cleave the AP site at the 5′ side, the repair intermediate containing 5′-deoxyribose phosphate component was processed in yeast cell extracts (80 μg) without ATP and dNTPs at 23°C for various times as indicated. (A) Repair in wild-type extracts. (B) Repair in rad27 mutant extracts. Repair products were analyzed by electrophoresis on a 20% denaturing polyacrylamide gel. The endonuclease IV-cleaved 5′ DNA fragment migrated at the 18mer position (lane 1). Under this electrophoresis condition, a labeled 3′ DNA fragment with or without the 5′-deoxyribose phosphate moiety migrated at the same position. Size markers in nucleotides are indicated on the sides.
strand break with a 5′ deoxyribose phosphate moiety. This repair intermediate was then further repaired in yeast cell-free extracts without ATP and dNTPs to avoid repair synthesis. After incubation in wild-type yeast extracts, DNA bands of 13–17 nt were evident (Fig. 4A), representing 3′ DNA fragments 2–6 nt shorter than the initial incised 3′ fragment. Hence, 1–5 nt were removed from the 5′ of the DNA nick, which presumably contained the 5′-deoxyribose phosphate moiety. In contrast, the corresponding 17mer and 16mer DNA bands were not detected and the formation of the 13–15mer DNA bands were reduced in rad27 mutant extracts (Fig. 4B). The source for the residual 13–15mer DNA bands in rad27 mutant extracts is not known. Thus, the removal of the 5′ deoxyribose phosphate moiety by nucleotide hydrolysis was deficient in rad27 mutant extracts. In the presence of ATP and dNTPs for repair synthesis, similar results were obtained (data not shown). These results suggest that Rad27 is required to process the 5′ DNA strand break after incision at an AP site by the Apn1 endonuclease.

MMS treatment generates significant AP sites in DNA, which result from the removal of methylated bases by 3-methyl adenine-DNA glycosylase (Mag1) (45) and spontaneous depurination of the labile N3-methyl adenine and N7-methyl guanine (46–48). In rad27 mutant cells, unprocessed DNA strand breaks may persist at some AP sites due to incisions by the residual Apn1 activity and deficiency in subsequent processing of the 5′ strand breaks. Hence, we conclude that the extreme sensitivity of rad27 mutant cells to MMS is caused by unprocessed DNA strand breaks accumulated during AP site repair, which can block replication and thus lead to lethality.

**BER of oxidative damage does not require Rad27**

BER of oxidative damage is initiated by DNA glycosylases with associated AP lyase activities (7,12). This mode of BER differs from the repair of uracil residues or AP sites in DNA in that a DNA strand break is formed 3′ to the AP site after the combined glycosylase and AP lyase reactions. Hence, AP site does not exist as a repair intermediate. The role of Rad27 in the repair of oxidative damage was investigated.

Osmium tetroxide-damaged DNA contains predominantly thymine glycols which are repaired by the Ntg proteins through the BER pathway in yeast cell-free extracts (11,14,36,37). Consistent with our previous results, BER repair synthesis (labeled by 32P) of osmium tetroxide-damaged DNA was readily detected in wild-type yeast extracts (Fig. 5A, lane 1). Such repair synthesis was not affected in the isogenic rad27 mutant extracts (Fig. 5A, lane 3). Proficient repair synthesis was also observed at 37°C (Fig. 5A, lane 3), a restrictive temperature for the growth of rad27 mutants (19,23). Since Rad27 possesses a flap endonuclease activity (21), it is conceivable that defects in this activity may lead to repair intermediates that cannot be ligated. To test this possibility, we examined the ligation step during BER of osmium tetroxide-damaged DNA. After *in vitro* repair, the plasmid DNA was directly loaded onto an agarose gel without linearization with *Hind* III restriction endonuclease. Repair products were separated by electrophoresis in the presence of ethidium bromide. Under this condition, the ligated repair products (closed circular DNA) were separated from the unligated repair products (nicked circular DNA) which migrated slower (Fig. 5B). As shown in Figure 5B (compare the closed circular DNA of lanes 1–3 with lanes 4–6, respectively), DNA ligation was not significantly affected without Rad27, regardless of the reaction temperatures. These results show that BER of osmium tetroxide-damaged DNA does not require Rad27 protein.

The effect of Rad27 on NER was also determined. Under the conditions used, repair of pUC18 DNA containing AAF adducts
is specifically mediated by the NER pathway in yeast cell-free extracts (37,38). In vitro NER was traced by \(^{32}\)P-labeling of the repair patch. In vitro NER was proficient in wild-type yeast extracts, but defective in NER-deficient rad10 or ssl2 mutant extracts (Fig. 6, lanes 1–4). In rad27 mutant extracts, repair synthesis was proficient (Fig. 6, lane 5). Furthermore, the DNA ligation step was also proficient, indicated by the observation that the majority of repaired products were in the ligated form (Fig. 6, lane 5), similar to repair in wild-type extracts (37). These results show that Rad27 protein does not play a role in NER. Hence, yeast Rad27 is specifically required for BER of AP sites in DNA.

**DISCUSSION**

There are two major modes of BER. One BER pathway is represented by the repair of oxidative damage which is initiated by a DNA glycosylase with associated AP lyase. AP sites formed after glycosylase action are immediately cleaved at their 3’ side by the AP lyase activity of the same protein. The other mode of BER is represented by the repair of uracil residues and AP sites in DNA, which does not involve an AP lyase. In yeast, the protein requirements for the two BER pathways have not been completely defined, although much is known about uracil BER (7,39). Using a yeast cell-free system, we have shown that BER of uracil residues in DNA is deficient in rad27 mutant extracts, whereas BER of thymine glycols and NER of AAF are not affected by the absence of Rad27 protein. The requirements for Rad27 protein in uracil BER *in vitro* are 2-fold. First, in the absence of Rad27 protein, the Apn1 endonuclease activity was significantly reduced. This led to deficient AP site incision which in turn resulted in deficient repair synthesis. Secondly, following Apn1 incision, hydrolysis of one or a few nucleotides (presumably containing the 5’-deoxyribose phosphate moiety) from the 5’ of the DNA strand break was deficient without Rad27 protein. Hence, Rad27 is specifically required for yeast BER of AP sites in DNA.

The precise molecular basis for the effect of Rad27 on Apn1 activity is presently unknown. Since APN1 gene is adjacent to RAD27 gene, rad27 deletion was carefully targeted ~40 bp downstream of the RAD27 stop codon, but ~230 bp upstream of the APN1 start codon (19). Thus, the APN1 gene is expected to be intact in this rad27 mutant strain. Our results and those of Reagan et al. (19) support this conclusion. It is possible that these two functionally-related genes may be coordinately regulated. Consistent with such a possibility, we observed a 3-fold reduction in APN1 mRNA in rad27 mutant cells as measured by quantitative RT–PCR. It is also possible that the stability and/or activity of Apn1 may be directly affected by Rad27 protein.

During BER of AP sites, DNA strand breaks are introduced by Apn1 incisions. Subsequent repair involves Rad27 to process the 5’ DNA strand breaks through nucleotide hydrolyses. Without Rad27, unprocessed DNA strand breaks may accumulate at some AP sites, which in turn can lead to lethality during DNA replication. This provides a molecular basis for the response of rad27 mutants to DNA damaging agents: highly sensitive to MMS, but not to ionizing radiation, and only slightly sensitive to UV (19). While the latter two agents cause significant DNA double strand breaks and bulky photoproducts, respectively, which are predominantly repaired by recombination and NER, respectively, MMS leads to AP sites in DNA. This mechanism of MMS sensitivity is further supported by the observation that deleting APN1 gene to prevent 5’ AP site incision largely restored the resistance of rad27 cells to MMS. The apn1 rad27 double mutant was slightly more sensitive to MMS than apn1 single mutant (Fig. 3). This may have resulted from the minor endonuclease activity of Apn2 (49). Consistent with this interpretation, the Apn2 endonuclease activity in yeast extracts is too low to be detected by our assays even in the absence of Apn1. Nevertheless, in the absence of Apn1, Apn2 appears to play a minor role in AP site repair *in vivo* (49).

In higher eukaryotes, it appears that the 5’-deoxyribose phosphate moiety derived from a natural AP site is mainly removed by the dRPase activity of DNA polymerase β, independent of FEN1 (8–10,31). Apparently, this polymerase β-dependent mechanism is not functional in yeast (41,42; X.Wu and Z.Wang, unpublished results). Our studies suggest that the 5’-deoxyribose phosphate component is likely removed in yeast by hydrolysis of 1–5 nt 3’ to the baseless sugar phosphate. This activity of Rad27 can occur in the absence of DNA repair synthesis. Since flap DNA structure is not possible without strand displacement synthesis, the removal of nucleotides from 5’ of the strand break by Rad27 must be mediated by its exonuclease activity. It is possible that Rad27 may also be involved in removing DNA flap structures that are formed by repair synthesis in the presence of 5’-deoxyribose phosphate moiety. Removal of the 5’ baseless sugar phosphate by the nuclease activities of FEN1 has been observed with modified AP sites in *Xenopus* and a human cell-free system (31,32).

The major DNA glycosylases for oxidative damage identified so far all contain an associated AP lyase activity. Thus, repair of oxidative damage involves 3’ AP site incision and subsequent removal of the 3’ baseless sugar phosphate component. Since we did not observe a significant defect during repair of thymine glycols induced by osmium tetroxide in rad27 mutant extracts, it is likely that repair of the 3’ baseless moiety may involve other protein(s) in addition to the endonuclease Apn1. Supporting this notion, we detected significant residual repair synthesis with osmium tetroxide-damaged DNA in *apn1* mutant extracts (7). In BER initiated by a glycosylase without associated AP lyase, removal of the 5’-deoxyribose phosphate component is a rate limiting step (7). Hence, repair of oxidative damage with AP lyase should effectively avoid competing against BER of other DNA damage such as uracil residues and AP sites at the same rate-limiting step. Diverting BER into two major repair pathways with two classes of DNA glycosylases would therefore enhance the overall repair efficiencies for various lesions in the genome.

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