Endogenous DNA abasic sites cause cell death in the absence of Apn1, Apn2 and Rad1/Rad10 in *Saccharomyces cerevisiae*

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In *Saccharomyces cerevisiae*, mutations in *APN1*, *APN2* and either *RAD1* or *RAD10* genes are synthetic lethal. In fact, *apn1 apn2 rad1* triple mutants can form microcolonies of ~300 cells. Expression of Nfo, the bacterial homologue of Apn1, suppresses the lethality. Turning off the expression of Nfo induces G₂/M cell cycle arrest in an *apn1 apn2 rad1* triple mutant. The activation of this checkpoint is *RAD9* dependent and allows residual DNA repair. The Mus81/Mms4 complex was identified as one of these back-up repair activities. Furthermore, inactivation of Ntg1, Ntg2 and Ogg1 DNA *N*-glycosylase/AP lyases in the *apn1 apn2 rad1* background delayed lethality, allowing the formation of minicolonies of ~10⁵ cells. These results demonstrate that, under physiological conditions, endogenous DNA damage causes death in cells deficient in Apn1, Apn2 and Rad1/Rad10 proteins. We propose a model in which endogenous DNA abasic sites are converted into 3'-blocked single-strand breaks (SSBs) by DNA *N*-glycosylases/AP lyases. Therefore, we suggest that the essential and overlapping function of Apn1, Apn2, Rad1/Rad10 and Mus81/Mms4 is to repair 3'-blocked SSBs using their 3'-phosphodiesterase activity or their 3'-flap endonuclease activity, respectively.

Keywords: abasic sites/Apn1, Apn2/cell death/Rad1/Rad10/*S.cerevisiae*

Introduction

Cellular DNA is damaged continuously by endogenous and exogenous reactive species. The outcome of DNA damage is generally adverse, contributing to ageing and oncogenic processes (Lindahl, 1993; Hoeijmakers, 2001). Apurinic/apyrimidinic (AP) sites are one of the most frequent lesions in DNA. AP sites can be formed by spontaneous hydrolysis of the *N*-glycosylic bond. It has been suggested that >10⁴ bases are lost per day per mammalian cell (Lindahl, 1993; Nakamura and Swenberg, 1999). AP sites are also formed as a consequence of the removal of modified bases by DNA *N*-glycosylases (Krokan *et al.*, 1997; Lindahl and Wood, 1999; Scharer and Jiricny, 2001). Moreover, AP sites induce the formation of single-strand breaks (SSBs) after cleavage by AP endonucleases or by DNA *N*-glycosylases/AP lyases (Krokan *et al.*, 1997; Scharer and Jiricny, 2001). AP

endonucleases cleave DNA at the 5' side of an AP site, yielding an SSB with a 3'-OH group. On the other hand, DNA *N*-glycosylases/AP lyases incise DNA at the 3' side of an AP site, yielding a 3'-blocked SSB with an α,β -unsaturated aldehyde, 4*R*-4-hydroxy-*trans*-2-pentenal (3'-dRP), moiety which cannot be used as substrate by DNA polymerases (Dempfle and Harrison, 1994). AP sites have to be repaired efficiently because of their potential cytotoxicity and mutagenicity (Loeb, 1985; Haracska *et al.*, 2001). Moreover, 3'-blocked SSBs can be converted into highly toxic double-strand breaks (DSBs) after DNA replication (Caldecott, 2001).

In the course of evolution, organisms have developed robust DNA repair mechanisms to minimize the deleterious effects of endogenous DNA damage (Friedberg *et al.*, 1995). The base excision repair (BER) pathway, mediated by AP endonucleases, is the primary defence against AP sites and 3'-blocked SSBs (Lindahl and Wood, 1999; Hoeijmakers, 2001). *Saccharomyces cerevisiae* possesses two AP endonucleases, namely Apn1 and Apn2. Apn1 is the major AP endonuclease activity; it shares extensive homology with *Escherichia coli* Nfo (endonuclease IV) (Dempfle and Harrison, 1994). Apn2 shares sequence homology with *E.coli* Xth (exonuclease III) and human APE1 and accounts for <10% of total AP endonuclease activity in *S.cerevisiae* (Johnson *et al.*, 1998; Bennett, 1999). Apn1 and Apn2 catalyse the hydrolytic cleavage of the phosphodiester backbone at the 5' side of an AP site, yielding an SSB with a 3'-OH group (Dempfle and Harrison, 1994; Unk *et al.*, 2000). Apn1 and Apn2 are also endowed with a 3'-phosphodiesterase activity removing 3'-blocking groups such as 3'-phosphate (3'-P), 3'-phosphoglycolate (3'-PGA) or 3'-dRP (Dempfle and Harrison, 1994; Unk *et al.*, 2001). *Saccharomyces cerevisiae apn1* mutants are moderately sensitive to the killing action of alkylating agents such as methyl methanesulfonate (MMS), whereas *apn1 apn2* double mutants are highly sensitive to MMS (Ramotar *et al.*, 1991; Johnson *et al.*, 1998; Bennett, 1999). Apn1-deficient strains also exhibit enhanced spontaneous mutation rates (Ramotar *et al.*, 1991). Furthermore, the mutator phenotype of an *apn1 apn2* double mutant is higher than that of an *apn1* single mutant (Bennett, 1999). Therefore, Apn1- and Apn2-deficient strains are viable and exhibit relatively mild phenotypes, which is unexpected, since AP sites are postulated to be the most abundant endogenous lesion in DNA. This might be explained by the presence of overlapping DNA repair pathways (Swanson *et al.*, 1999; Gellon *et al.*, 2001). Two studies point to nucleotide excision repair (NER) as a candidate. In yeast, mutations in the NER genes, such as *RAD1*, *RAD2*, *RAD4* and *RAD10*, and in the BER gene *APN1* are synergistic with respect to killing by MMS, a methylating agent that generates AP sites in DNA (Xiao and Chow, 1998).

Fig. 1. Synthetic lethality of *apn1 apn2* with *rad1* but not with *rad14* in *S. cerevisiae*. (A) The haploid strains BG3 (*apn1 apn2*) and FF181482 (*rad1*) were crossed (Table I). After sporulation of diploids, tetrads were dissected on YPD plates. The spore clones obtained were genotyped by replica plating on selective media. The genotype of inviable spores was inferred from segregation patterns. The squares surround the triple mutant *apn1 apn2 rad1*. Microscopic analysis ($\times 40$) shows that these squares surround microcolonies. Photographs were taken after 4 days at 30°C. (B) The haploid strains BG3 (*apn1 apn2*) and BG35 (*rad14*) were crossed. The cross was analysed as before. (C) Tetrads resulting from the *apn1 apn2* $\times$ *rad1* cross were dissected and the growth of each spore was followed by microscopy. Growth of a wild-type and of an *apn1 apn2 rad1* triple mutant is shown. (D) Number of cells per wild-type and triple mutant colony. The wild-type curve is the average of three colonies. The triple mutant curve is the average of seven colonies. (E) A representative *apn1 apn2 rad1* microcolony after 4 days at 30°C.

Another study shows that mutations in NER genes *RAD2*, *RAD4* and *RAD14* strongly increase the sensitivity to MMS of strains harbouring mutations in BER genes *APN1* and *APN2* (Torres-Ramos *et al.*, 2000). Although very sensitive to MMS, these mutants are viable (Torres-Ramos *et al.*, 2000). In mammalian cells, mutants deficient in the major AP endonuclease APE1 are embryonic lethal, which may suggest that repair of AP sites is essential in mice (Meira *et al.*, 2001).

In this study, we show that mutations in *APN1*, *APN2* and either *RAD1* or *RAD10* genes are synthetic lethal in *S. cerevisiae*. In contrast, an *apn1 apn2 rad14* triple mutant is viable. The results ascribed a novel activity, independent of NER, to the Rad1/Rad10 heterodimer. The present study also involves the Rad9 checkpoint protein and the Mus81/Mms4 heterodimer in the repair of endogenous DNA damage. We propose a model in which endogenous AP sites are converted into 3'-blocked SSBs by DNA *N*-glycosylases/AP lyases. Furthermore, we suggest that the essential and overlapping function of Apn1, Apn2 and Rad1/Rad10 is to repair 3'-blocked SSBs using their 3'-phosphodiesterase activity or its 3'-flap endonuclease activity, respectively.

Results

Synthetic lethality of *apn1 apn2* with either *rad1* or *rad10* but not with *rad14*

In *S. cerevisiae*, *apn1 apn2* double mutants only present a modest spontaneous mutator phenotype, suggesting the involvement of other repair pathways in the removal of endogenous AP sites in DNA. To investigate the role of NER in the removal of endogenous DNA damage, *rad1* or *rad14* strains were crossed to *apn1 apn2* double mutants. In the *rad1* $\times$ *apn1 apn2* cross, a high degree of spore inviability was observed (Figure 1A). Spore clones recovered from 30 tetrads were genotyped by replica plating to appropriate media. No *apn1 apn2 rad1* triple mutant was obtained (15 triple mutants expected). In addition, all the 14 inviable spores obtained in this cross were *apn1 apn2 rad1* triple mutants. The same result was observed with the *rad10* $\times$ *apn1 apn2* cross, showing lethality of the *apn1 apn2 rad10* triple mutant (12 tetrads analysed, no triple mutant obtained) (data not shown). In contrast, the *rad14* $\times$ *apn1 apn2* cross does not show spore inviability (Figure 1B). The spore clones were also genotyped to confirm the viability of the *apn1 apn2*

rad14 triple mutant (15 tetrads analysed, seven triple mutants obtained).

Although unable to form visible colonies, the *apn1 apn2 rad1* triple mutants can form microcolonies. Figure 1C shows that 8 h after dissection, wild-type and *apn1 apn2 rad1* cells have similar aspects. After 16 h, the *apn1 apn2 rad1* triple mutant exhibits a slow growth rate and cells enlarge in size compared with the wild type. After 26 h, the triple mutant clone is composed of a small number of cells, of which ~90% are 'large budded' cells, suggesting a cell cycle arrest in G₂ (Figure 1C). The number of cells per wild-type and triple mutant colony was measured as a function of time. Figure 1D shows that *apn1 apn2 rad1* triple mutants grow very slowly compared with the wild type. After 4 days, the *apn1 apn2 rad1* triple mutant has generated a microcolony of ~300 cells (Figure 1E), whereas a wild-type colony contains >10⁷ cells. These results show that *apn1 apn2 rad1* spores can undergo a limited number of generations before death. The viability of *apn1 apn2 rad14* triple mutants demonstrates that the inactivation of the NER pathway is not sufficient to generate the lethality. This also indicates a specific role for the Rad1/Rad10 complex.

Endogenous DNA damage is lethal in *apn1 apn2 rad1* triple mutants

The known functions of Apn1, Apn2 and Rad1/Rad10 point to DNA damage at the origin of the lethality of *apn1 apn2 rad1* triple mutants. To test this hypothesis, we have expressed an AP endonuclease, Nfo (endonuclease IV) from *E.coli*, into an *apn1 apn2 rad1* triple mutant of *S.cerevisiae*. The *nfo* gene was cloned under the control of the galactose-inducible promoter *GAL1* in the centromeric plasmid p414GAL1, yielding p414GAL1-*nfo*. Figure 2 shows that, in the presence of galactose, the expression of Nfo from p414GAL1-*nfo* suppresses the hypersensitivity of the *apn1 apn2* double mutant with respect to the killing effect of MMS. In contrast, in the presence of glucose, cells hosting p414GAL1-*nfo* are still hypersensitive to MMS (Figure 2). This result shows that Nfo is functional in yeast and its expression can be regulated by the growth medium (Ramotar and Demple, 1996).

To construct an *apn1 apn2 rad1* triple mutant expressing Nfo, a diploid (BG83: *apn1/APN1 apn2/APN2 rad1/RAD1*) strain was transformed with p414GAL1-*nfo*. This latter diploid was sporulated on galactose and a haploid triple mutant *apn1 apn2 rad1/p414GAL1-nfo* strain was isolated (BG84/p414GAL1-*nfo*). Figure 3A shows that BG84/p414GAL1-*nfo* can grow on YNBGal plates whereas it does not grow on YNBD plates. Similar results were obtained using p414GAL1-derived constructs expressing Apn1 or Apn2 from *S.cerevisiae* or Xth (exonuclease III) from *E.coli* (data not shown). Figure 3B shows that BG84/p414GAL1-*nfo* cultures progressively stop growing when shifted from galactose to glucose. Therefore, the mechanisms of cell death in *apn1 apn2 rad1* triple mutants can be investigated in liquid cultures. Fluorescence-activated cell sorting (FACS) analysis of BG84/p414GAL1-*nfo* cell cultures shows a progressive reduction of the fraction of cells in G₁ as a function of time after the shift from galactose to glucose (Figure 3C, bottom right). It should be noted that BG84/p414GAL-*nfo* cell cultures grown in galactose present a deficit in G₁ cells

Fig. 2. The bacterial AP endonuclease Nfo complements the yeast *apn1 apn2* double mutant hypersensitivity to killing by MMS. BG3 (*apn1 apn2*) was transformed with p414GAL1-*nfo*, a centromeric plasmid containing the *nfo* gene of *E.coli* placed under the control of a galactose-inducible promoter. BG3 cells harbouring p414-GAL1 or p414GAL1-*nfo* were grown in either YNBD or YNBGal, and exposed to increasing amounts of MMS. Experimental points are the average of three independent experiments.

compared with controls (Figure 3C, bottom left and upper part). After 24 h in glucose, the FACS results show a very broad distribution of cells, which probably reflects cell death (data not shown). Finally, BG84/p414GAL1-*nfo* cells grown for 9 h in glucose were stained with 4',6-diamidino-2-phenylindole (DAPI) and observed by fluorescence microscopy. Figure 3D shows large budded cells with the nucleus localized at the bud neck. This is characteristic of cells arrested at the G₂/M checkpoint and more specifically at the pre-anaphase stage (Paulovich *et al.*, 1997; Toczyski *et al.*, 1997). The ability of a bacterial AP endonuclease to suppress the synthetic lethality of mutations in *APN1*, *APN2* and *RAD1* led us to conclude that endogenous DNA damage causes cell death in the absence of Apn1, Apn2 and Rad1/Rad10. In addition, endogenous DNA damage induces a G₂/M checkpoint, which presumably delays cell death of *apn1 apn2 rad1* triple mutant strains.

Endogenous DNA damage induces a RAD9-dependent G₂/M checkpoint allowing residual repair in *apn1 apn2 rad1* triple mutants

A G₂/M checkpoint in response to various DNA-damaging agents is genetically controlled by several genes, *RAD9* being one of them in *S.cerevisiae* (Weinert and Hartwell, 1988; Toczyski *et al.*, 1997). To investigate the role of the *RAD9* gene in the G₂/M checkpoint induced by endogenous DNA damage, we generated an *apn1 apn2 rad1 rad9* quadruple mutant strain. A diploid strain (BG137: *apn1/APN1 apn2/APN2 rad1/RAD1 rad9/RAD9*) was sporulated and, after dissection, a high degree of spore inviability was observed (119 tetrads analysed, 65 inviable spores). After 4 days, microscopic analysis revealed two classes of microcolonies. Class 1 are composed of ~300 large cells similar to the *apn1 apn2 rad1* triple mutants (Figure 4A, left), whereas class 2 are composed of ~20 normal size cells (average of eight colonies) (Figure 4A, right). Tetrads were analysed and the genotype of the microcolonies was inferred from segregation patterns. All

Fig. 3. Synthetic lethality of mutations in *APN1*, *APN2* and *RAD1* genes is due to endogenous DNA damage. (A) Haploid strains BG3/p414GAL1-*nfo* (*apn1 apn2*/p414GAL1-*nfo*) and BG84/p414GAL1-*nfo* (*apn1 apn2 rad1*/p414GAL1-*nfo*) (Table I) were grown in galactose-containing medium to an $OD_{600} = 1.0$ and plated onto glucose- (YNBD) and galactose-containing (YNBGal) plates. (B) The same two cultures were washed in sterile water and diluted to $OD_{600} = 0.1$ in fresh YNBD or YNBGal. After dilution in the appropriate medium ($t = 0$), the growth of the four cultures was measured as a function of time. (C) FACS analysis of aliquots of the four cultures described in (B) as a function of time after the shift from galactose to glucose. (D) DAPI staining of BG84/p414GAL1-*nfo* cells after 9 h in YNBD.

class 1 microcolonies tested (6/6) were *apn1 apn2 rad1* triple mutants, whereas all class 2 tested (8/8) were *apn1 apn2 rad1 rad9* quadruple mutants. The fact that Rad9-deficient (class 2) microcolonies contain, nearly exclusively, normal size cells (Figure 4A, right; data not shown) suggests that in those cells the G₂/M checkpoint is not activated. FACS was used to assess the role of *RAD9* in the activation of the G₂/M checkpoint by endogenous DNA damage. For these studies, we isolated a haploid *apn1 apn2 rad1 rad9*/p414GAL1-*nfo* (BG138/p414GAL1-*nfo*)

strain. FACS shows that BG138/p414GAL1-*nfo* behaves differently from BG84/p414GAL1-*nfo*. In the presence of galactose, the Rad9-deficient strain presents a fraction of cells in G₁ similar to that of a wild-type strain (Figure 4B, left), whereas the Rad9-proficient strain exhibits a deficit of cells in G₁ (Figure 3C, bottom left). FACS analysis also shows that 3, 6 and 9 h after shift from galactose to glucose, the *apn1 apn2 rad1 rad9*/p414GAL1-*nfo* population still contains a significant fraction of cells in G₁ (Figure 4B, right). Together, the results suggest that the

Fig. 4. Activation of a G_2/M checkpoint by endogenous DNA damage is *RAD9* dependent. (A) BG3 (*apn1 apn2*) and BG88 (*rad1 rad9*) strains were crossed. Tetrads were dissected after sporulation of diploids on YPD plates. The genotype of spores was determined by replica plating on selective media and by PCR. The genotype of inviable spores was inferred from segregation patterns. The microscopic appearance of class 1 (*apn1 apn2 rad1*) and class 2 (*apn1 apn2 rad1 rad9*) microcolonies after 4 days at 30°C is shown. (B) The BG138/p414GAL1-*nfo* (*apn1 apn2 rad1 rad9/p414GAL1-nfo*) strain was grown in YNBGal and subsequently diluted in YNBD or YNBGal at $t = 0$ as described. FACS analysis of aliquots of the two cultures was performed as described (Figure 3).

activation of the G_2/M checkpoint by unrepaired endogenous DNA damage is *RAD9* dependent. Furthermore, the fact that Rad9-deficient microcolonies contain fewer cells than Rad9-proficient ones indicates that activation of the G_2/M checkpoint allows some residual DNA repair.

Inactivation of the Mus81/Mms4 endonuclease causes early death of *apn1 apn2 rad1* triple mutants

A recent study reported that Mus81 and Mms4 proteins form a heterodimeric structure-specific endonuclease that cleaves branched DNA with a 3'-single-stranded extension (Kaliraman *et al.*, 2001). Mus81 and Mms4 are conserved proteins related to the Rad1/Rad10 endonuclease. The overlapping substrate specificities of Rad1/Rad10 and Mus81/Mms4 endonucleases led us to hypothesize that Mus81/Mms4 could be a back-up repair activity for endogenous DNA damage in the *apn1 apn2 rad1* triple mutant. To test this hypothesis, a diploid strain (BG156: *apn1/APN1 apn2/APN2 rad1/RAD1 mus81/MUS81*) was sporulated and dissected. We analysed 36 tetrads, and 21 spores were inviable. After 4 days, two classes of microcolonies were observed: class 1 is composed of ~300 large cells similar to the *apn1 apn2 rad1* triple mutant (Figure 5A, left), and class 3 is composed of 17 large cells presumably blocked at the G_2/M checkpoint (Figure 5A, right). Tetrads were analysed and the genotypes were inferred from segregation patterns. The results show that the *apn1 apn2 mus81* triple mutant is

Fig. 5. Inactivation of the Mus81/Mms4 endonuclease causes early death of an *apn1 apn2 rad1* triple mutant. (A) BG3 (*apn1 apn2*) and BG154 (*mus81 rad1*) strains were crossed. Tetrads were dissected after sporulation of diploids on YPD plates. The genotype of spores was determined by replica plating on selective media and by PCR. The genotype of inviable spores was inferred from the segregation patterns. The microscopic appearance of class 1 (*apn1 apn2 rad1*) and class 2 (*apn1 apn2 mus81 rad1*) microcolonies after 4 days at 30°C is shown. (B) The average number of cells of the three classes of microcolonies was estimated after 4 days at 30°C. All numbers of cells are means $\pm$ SD from at least three independent colonies.

viable. Furthermore, analyses of microcolonies revealed that all class 1 microcolonies tested (3/3) were *apn1 apn2 rad1* triple mutants as expected. On the other hand, all class 3 microcolonies tested (6/6) were *apn1 apn2 rad1 mus81* quadruple mutants. Figure 5B shows the average number of cells for the three classes of microcolonies observed in this study. The comparison of the number of cells in class 1 and class 3 microcolonies strongly suggests that Mus81/Mms4 activity delayed death of *apn1 apn2 rad1* triple mutants. Therefore, the Mus81/Mms4 complex is one of the back-up repair activities that are acting during the Rad9-dependent cell cycle arrest, as suggested before. The role of the Mus81/Mms4 heterodimer again points to 3'-flap endonuclease activity for the repair of endogenous DNA damage.

Inactivation of Ntg1, Ntg2 and Ogg1 AP lyases delays death of *apn1 apn2 rad1* triple mutants

The substrate specificity of Apn1 and Apn2 suggests that AP sites are responsible for cell death in *apn1 apn2 rad1* mutants. However, *S.cerevisiae* possesses three DNA N-glycosylases/AP lyases, Ntg1, Ntg2 and Ogg1, which are able to nick DNA at the 3' side of an AP site in DNA. Therefore, these proteins convert AP sites into 3'-blocked (3'dRP) SSBs (Scharer and Jiricny, 2001). Thus, inacti-

Fig. 6. Inactivation of the DNA *N*-glycosylases/AP lyases Ntg1, Ntg2 and Ogg1 results in enhanced survival. (A) BG3 (*apn1 apn2*) and CC893 (*ntg1 ntg2 ogg1 rad1*) strains were crossed. Tetrads were dissected after sporulation of diploids on YPD plates. Spore genotype was determined by replica plating on selective media and by PCR. A selection of tetrads containing a minicolony (A1) and a microcolony (B2) is presented. (B) BG3 (*apn1 apn2*) and BG81 (*apn1 apn2 ntg1 ntg2 ogg1*) cells were treated with MMS. Samples were diluted and plated on YPD solid medium to assess cell viability.

vation of *NTG1*, *NTG2* and *OGG1* should prevent the cleavage of AP sites in DNA and consequently reduce the number of 3'-dRP SSBs. To investigate the relative impact of AP sites and 3'-blocked SSBs in cell death induced by endogenous DNA damage, we constructed yeast strains deficient in Apn1, Apn2, Rad1 and all three AP lyases. Thus, BG3 (*apn1 apn2*) was crossed with CC893 (*ntg1 ntg2 ogg1 rad1*). After sporulation of the resulting diploid, 206 tetrads were dissected, yielding 14 minicolonies in addition to microcolonies (Figure 6A). After 4 days, minicolonies contain $\sim 10^5$ cells. The genotype of eight minicolonies was determined, and all of them were *apn1 apn2 rad1 ntg1 ntg2 ogg1* sextuple mutants. Although able to form visible colonies, the sextuple mutant cannot grow in liquid cultures. However, inactivation of all three AP lyases greatly enhances the number of generations that can undergo an *apn1 apn2 rad1* triple mutant. This last result strongly suggests that 3'-blocked (3'-dRP) SSBs are more toxic than the initial AP site in DNA. To support this hypothesis, we have compared the sensitivity to MMS of an *apn1 apn2* double mutant deficient or not in the three AP lyases. Figure 6B shows that inactivation of Ntg1, Ntg2 and Ogg1 protects the *apn1 apn2* double mutant from the killing action of MMS, again indicating that 3'-blocked SSBs are more toxic than intact AP sites.

Discussion

Reactive oxygen species attacks and the hydrolytic decomposition of DNA have been suggested to be at the origin of the majority of endogenous DNA lesions (Cadet *et al.*, 1997). AP sites are probably the most abundant endogenous lesion in DNA and have been shown to be cytotoxic and mutagenic (Lindahl, 1993; Nakamura and Swenberg, 1999; Haracska *et al.*, 2001). However, Apn1- and Apn2-deficient strains are viable and present only a weak mutator phenotype (Bennett, 1999). This might be explained by the presence of overlapping DNA repair pathways (Swanson *et al.*, 1999; Gellon *et al.*, 2001).

In this study, we show that mutations in *APN1*, *APN2* and either *RAD1* or *RAD10* are synthetic lethal. However, the *apn1 apn2 rad1* triple mutant can undergo a limited number of generations, allowing the formation of microcolonies. It should be noted that *apn1 rad1* and *apn2 rad1* double mutants are viable. In addition, we confirm that an *apn1 apn2 rad14* triple mutant is also viable (Torres-Ramos *et al.*, 2000; Leroy *et al.*, 2001). These results clearly show that inactivation of NER is not the cause of the cell death in the *apn1 apn2 rad1* triple mutant. Therefore, the synthetic lethality of the triple mutant is due to a specific function of the Rad1/Rad10 complex. Although not sufficient, we cannot exclude the possibility that inactivation of NER is required to cause cell death. The lethality could be due to the accumulation of unrepaired DNA damage or to another essential function that is not directly related to DNA repair. The possibility of suppressing the lethality of the *apn1 apn2 rad1* triple mutant by Nfo, an AP endonuclease from *E. coli*, points to endogenous DNA damage as being at the origin of cell death. The results also show that the inactivation of the AP lyases, Ntg1, Ntg2 and Ogg1, allows more generations before cell death in the *apn1 apn2 rad1* background. This result is consistent with the idea that a 3'-blocked (3'-dRP) SSB resulting from cleavage of an AP site by an AP lyase is more toxic than the AP site itself. A recent study reports synthetic lethality of *apn1 apn2 tpp1 rad52* quadruple mutants which is associated with the accumulation of a 3'-P-blocked SSB (Vance and Wilson, 2001). *TPP1* encodes a 3'-phosphatase function able to release 3'-blocked (3'-P) SSBs in DNA (Vance and Wilson, 2001). However, the *apn1 apn2 tpp1* triple mutant is viable, which suggests that 3'-P is a minor class 3'-blocked SSB compared with 3'-dRP. Alternatively, another efficient repair pathway, presumably Rad1/Rad10 dependent, remains in these cells. Therefore, we propose that the lethality of the *apn1 apn2 rad1* triple mutant is due to the accumulation of endogenous DNA damage, mostly 3'-blocked (3'-dRP) SSBs, which are substrates of Apn1, Apn2 and Rad1/Rad10.

In yeast, Apn1 and Apn2 are involved primarily in the BER of AP sites and 3'-blocked SSBs in DNA (Dempfle and Harrison, 1994; Unk *et al.*, 2001). The Rad1 and Rad10 proteins of *S. cerevisiae* are indispensable for NER and they are also required for an additional mitotic recombination pathway (Fishman-Lobell and Haber, 1992; Ivanov and Haber, 1995; Saparbaev *et al.*, 1996). The Rad1/Rad10 complex recognizes DNA duplex-single strand junctions and cleaves the 3'-single-strand extension near the junction (Bardwell *et al.*, 1994; Habraken *et al.*, 1994; Davies *et al.*, 1995). Here, we suggest that Rad1/

Fig. 7. A model for cell death induced by endogenous DNA damage in *S. cerevisiae*. **(A)** Pathways yielding viable colonies. Endogenous stress generates AP sites in DNA. In wild-type cells, AP sites are repaired primarily by Apn1 and Apn2. NER can act as a back-up repair pathway. However, some of the AP sites are converted by DNA N-glycosylases/AP lyases (Ntg1, Ntg2, Ogg1) into 3'-blocked (3'-dRP) SSBs which are also repaired by Apn1 and Apn2. In *apn1 apn2* double mutants, one expects the accumulation of 3'-blocked SSBs. The model suggests an alternative repair pathway mediated by the Rad1/Rad10 complex through its 3'-flap endonuclease activity. **(B)** Pathways yielding inviable microcolonies. In the absence of Apn1, Apn2 and Rad1/Rad10 proteins, 3'-blocked SSBs persist and cause DSBs and replication fork collapse. DSBs can induce the activation of a G₂/M checkpoint that is *RAD9* dependent. Cell cycle arrest can allow residual repair by the Mus81/Mms4 3'-flap endonuclease, yielding class 1 (300 large cells). The absence of Mus81/Mms4 results in class 3 microcolonies (17 large cells). In the absence of Rad9 (dotted line), the G₂/M checkpoint is not activated, resulting in class 2 microcolonies (20 normal size cells). In all cases, the accumulation of DSBs will cause DNA degradation and cell death.

Rad10 uses its 3'-flap endonuclease activity to release 3'-blocked termini in DNA. This process involves the formation of a single-stranded DNA tail, possibly driven by a DNA helicase, with a 3'-dRP end. This last structure should be a substrate of Rad1/Rad10 (Bardwell *et al.*, 1994). This study also shows that Mus81/Mms4, another structure-dependent endonuclease acting at a 3'-single-stranded DNA extension, delayed cell death of the *apn1 apn2 rad1* triple mutant. The results show that *apn1 apn2 rad1* triple mutants can undergo about eight generations after dissection whereas *apn1 apn2 rad1 mus81* quadruple mutants can only undergo about four generations. These data again point to 3'-flap endonucleases being involved in the repair of endogenous DNA damage.

The results reported in this study are summarized in Figure 7. Endogenous stress induces a variety of types of DNA damage, AP sites being the most abundant. In a wild-type strain, AP sites are repaired primarily by Apn1, Apn2 and NER as a back-up. In the absence of Apn1 and Apn2, AP sites are processed mostly by Ntg1, Ntg2 and Ogg1 or undergo chemical cleavage, yielding 3'-blocked (3'-drp) SSBs which accumulate in the cell. Such lesions are substrates of the Rad1/Rad10 heterodimer with the Mus81/Mms4 heterodimer as a back-up, regenerating 3'-OH

termini. In the absence of *Apn1*, *Apn2* and *Rad1/Rad10*, a growing number of 3'-blocked SSBs persist in DNA and lead to replication fork collapse in which the SSB is converted into a DSB (Flores-Rozas and Kolodner, 2000; Caldecott, 2001). In *apn1 apn2 rad1* triple mutants, endogenous DNA damage activates a *RAD9*-dependent G₂/M checkpoint allowing some residual repair process. In the present study, we identified *Mus81/Mms4* as one of these back-up repair activities. It has been shown that stalled replication forks can restart by a *Rad52*-dependent pathway, which probably occurs in the *apn1 apn2 rad1* triple mutant during cell cycle arrest (Flores-Rozas and Kolodner, 2000). The role of *RAD52* is also suggested by the synthetic lethality of mutations in *APN1*, *APN2*, *TPP1* and *RAD52* (Vance and Wilson, 2001). Our scheme for cell death by endogenous DNA damage can be compared with that observed in the *cdc13-1* mutant that accumulates single-stranded DNA at the restricted temperature (Garvik *et al.*, 1995). However, the *cdc13-1* mutant can only form microcolonies of ~10 cells, whereas those of the *apn1 apn2 rad1 mus81* quadruple mutant contain ~17 cells. This last result strongly suggests that endogenous DNA AP sites occur very rapidly in the genome and cause very early cell death in the absence of DNA repair functions.

Table I. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Reference
FF18733	Mat a , <i>leu2-3-112</i> , <i>trp1-289</i> , <i>his7-2</i> , <i>ura3-52</i> , <i>lys1-1</i>	F.Fabre
FF18734	Mat α , <i>leu2-3-112</i> , <i>trp1-289</i> , <i>his7-2</i> , <i>ura3-52</i> , <i>lys1-1</i>	F.Fabre
BG1	FF18733 with <i>apn1Δ::URA3</i>	D.Ramotar
BG2	FF18733 with <i>apn2Δ::kanMX6</i>	This study
BG3	FF18733 with <i>apn1Δ::URA3</i> , <i>apn2Δ::kanMX6</i>	This study
BG3/p414GAL1- <i>nfo</i>	FF18733 with <i>apn1Δ::URA3</i> , <i>apn2Δ::kanMX6</i> with p414GAL1- <i>nfo</i>	This study
FF181482	FF18734 with <i>rad1Δ::LEU2</i>	F.Fabre
BG35	FF18734 with <i>rad14Δ::LEU2</i>	This study
BG83	Diploid <i>apn1Δ::URA3/APN1</i> , <i>apn2Δ::kanMX6/APN2</i> , <i>rad1Δ::LEU2/RAD1</i>	This study
BG83/p414GAL1- <i>nfo</i>	Diploid <i>apn1Δ::URA3/APN1</i> , <i>apn2Δ::kanMX6/APN2</i> , <i>rad1Δ::LEU2/RAD1</i> with p414GAL1- <i>nfo</i>	This study
BG84/p414GAL1- <i>nfo</i>	FF18734 with <i>apn1Δ::URA3</i> , <i>apn2Δ::kanMX6</i> , <i>rad1Δ::LEU2</i> with p414GAL1- <i>nfo</i>	This study
BG5	FF18733 with <i>rad9Δ::kanMX6</i>	This study
BG88	FF18734 with <i>rad1Δ::LEU2</i> , <i>rad9Δ::kanMX6</i>	This study
BG137	Diploid <i>apn1Δ::URA3/APN1</i> , <i>apn2Δ::kanMX6/APN2</i> , <i>rad1Δ::LEU2/RAD1</i> , <i>rad9Δ::kanMX6/RAD9</i>	This study
BG137/p414GAL1- <i>nfo</i>	Diploid <i>apn1Δ::URA3/APN1</i> , <i>apn2Δ::kanMX6/APN2</i> , <i>rad1Δ::LEU2/RAD1</i> , <i>rad9Δ::kanMX6/RAD9</i> with p414GAL1- <i>nfo</i>	This study
BG138/p414GAL1- <i>nfo</i>	FF18733 with <i>apn1Δ::URA3</i> , <i>apn2Δ::kanMX6</i> , <i>rad1Δ::LEU2</i> , <i>rad9Δ::kanMX6</i> with p414GAL1- <i>nfo</i>	This study
FF181659	FF18733 with <i>mus81Δ::kanMX6</i>	F.Fabre
BG154	FF18734 with <i>mus81Δ::kanMX6</i> , <i>rad1Δ::LEU2</i>	This study
BG156	Diploid <i>apn1Δ::URA3/APN1</i> , <i>apn2Δ::kanMX6/APN2</i> , <i>rad1Δ::LEU2/RAD1</i> , <i>mus81Δ::kanMX6/MUS81</i>	This study
CC893	FF18733 with <i>rad1Δ::LEU2</i> , <i>ntg1Δ::URA3</i> , <i>ntg2Δ::TRP1</i> , <i>ogg1Δ::LEU2</i>	D.Thomas
BG112	Diploid <i>apn1Δ::URA3/APN1</i> , <i>apn2Δ::kanMX6/APN2</i> , <i>rad1Δ::LEU2/RAD1</i> , <i>ntg1Δ::URA3/NTG1</i> , <i>ntg2Δ::TRP1/NTG2</i> , <i>ogg1Δ::LEU2/OGG1</i>	This study
BG81	FF18734 with <i>apn1Δ::URA3</i> , <i>apn2Δ::kanMX6</i> , <i>ntg1Δ::URA3</i> , <i>ntg2Δ::TRP1</i> , <i>ogg1Δ::LEU2</i>	This study

Materials and methods

Yeast culture and genetic procedures

Yeast strains were grown at 30°C in YP or YNB media supplemented with appropriate amino acids and bases and 2% glucose (YPD and YNBD) or 2% galactose (YPGal and YNBGal). All media, including agar, were from Difco. Pre-sporulation and sporulation procedures were performed as described (Resnick *et al.*, 1983). Micromanipulation and dissection of asci were performed using a Singer MSM System as described (Sherman and Hicks, 1991).

Yeast strains and plasmids

Saccharomyces cerevisiae strains used in this study are listed in Table I. All yeast strains are derivatives of the wild-type strain FF18733 (mat **a**, *leu2-3-112*, *trp1-289*, *his7-2*, *ura3-52*, *lys1-1*). *APN1*, *APN2* and *RAD9* gene deletions were produced by a PCR-mediated one-step replacement technique (Baudin *et al.*, 1993). *RAD1* and *RAD14* gene disruptions were performed using plasmids pWJ163 (from R.Rothstein) and pBM190 (Bankmann *et al.*, 1992), respectively. *NTG1*, *NTG2* and *OGG1* gene disruptions were described previously (Thomas *et al.*, 1997; Gellon *et al.*, 2001). All disruptions were confirmed by PCR on genomic DNA. To construct plasmid p414GAL1-*nfo*, the *nfo* gene of *E. coli* was amplified by PCR from genomic DNA with ENDOIVBam5 (5'-GCGGATCCCA-TGAAATACATTGGAGCGC-3') and ENDOIVEco3 (5'-GCGAATTC-TCAGGCTACCGCTTTTTCAG-3') as primers. The PCR product was digested by *Bam*HI and cloned into p414GAL1 (Mumberg *et al.*, 1994), previously digested by *Sma*I and *Bam*HI, yielding p414GAL1-*nfo*. Yeast strains were transformed with p414GAL1 or p414GAL1-*nfo* after lithium acetate treatment (Gietz *et al.*, 1992). BG83, BG137, BG156 and BG112 are diploid strains obtained by crossing BG3 (*apn1 apn2*) and FF181482 (*rad1*), BG88 (*rad1 rad9*), BG154 (*mus81 rad1*) or CC893 (*ntg1 ntg2 ogg1 rad1*) (Table I). The haploid strains BG84/p414GAL1-*nfo* and BG138/p414GAL1-*nfo* were isolated by tetrad dissection after sporulation on YPGal of diploids BG83/p414GAL1-*nfo* and BG137/p414GAL1-*nfo* strains, respectively.

FACS analysis and DAPI staining

Flow cytometry analyses were carried out with a FACScalibur (Beckton-Dickinson). For FACS analysis, yeast cells were grown overnight at 30°C in YNBGal and diluted to OD₆₀₀ = 0.1 in fresh YNBGal or YNBD (*t* = 0). After 3, 6 and 9 h, 10⁷ cells were harvested, fixed in 70% ethanol, washed in phosphate-buffered saline, incubated with 1 mg/ml RNase, centrifuged

and resuspended in 50 µg/ml propidium iodide. For DAPI staining, 10⁷ cells were fixed in 70% ethanol, centrifuged and resuspended in 0.04 ng/ml DAPI. Cells were analysed on a Zeiss Axiophot 2 microscope.

Determination of the genotype of micro- and minicolonies

The genotype of inviable spores was inferred from the segregation pattern of the three viable spores. The three colony spores of tetrads containing a micro- or a minicolony were grown on YPD overnight and genomic DNA was extracted with the Dneasy™ tissue Kit (Qiagen). PCRs were performed to determine the disruption of *OGG1*, *APN1*, *NTG1*, *RAD1* and *MUS81* genes with the following primers: OLSEB2 (5'-CTTTCT-CCACAAGGCATCC-3') and OLSEB6 (5'-CATTAATCTAATATGG-TCGAGTCT-3'), APN25 (5'-GGGGATGCCCTCGACACCTAGC-3') and APN232 (5'-AGGATCCTTATTCTTTCTTAGCTTCCTC-3'), NTG1-flank5 (5'-GCAGTTACAGTCACAGTCACAGCC3') and NTG1-flank3 (5'-GGCTCTGATTGGTGTCTGTATG-3'), RAD1-flank5 (5'-TCGGGA-CGAGTAACTTTTGTCTG-3') and RAD1-flank3 (5'-CATGTCTAAC-TTAAACATATACGGTTCG-3'), and KANR606 (5'-ACGGAATTT-ATGCCTCTTCCG-3') and MUS81-flank3 (5'-CGTACCTACCATTGA-TGAGTTGTCAAGTGGC-3').

Analysis of microcolonies

The number of cells per microcolony was counted by microscopic observation. Alternatively, the number of cells per colony was estimated after resuspension in sterile water and counting using a Malassez cell.

MMS sensitivity

Yeast cells were grown in YPD at 30°C to OD₆₀₀ = 1.0 and resuspended in sterile water. Appropriate dilutions of MMS (Sigma) were added for 20 min at 30°C with agitation. The reaction was terminated by adding 1 vol. of 10% sodium thiosulfate. To assess cell viability after treatment, appropriate dilutions of cell suspensions were plated on appropriate media and allowed to grow for 3 days at 30°C.

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