Silent repair accounts for cell cycle specificity in the signaling of oxidative DNA lesions

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Reactive oxygen species are the most important source of DNA lesions in aerobic organisms, but little is known about the activation of the DNA checkpoints in response to oxidative stress. We show that treatment of yeast cells with sublethal concentrations of hydrogen peroxide induces a Mec1-dependent phosphorylation of Rad53 and a Rad53-dependent cell cycle delay specifically during S phase. The lack of Rad53 phosphorylation after hydrogen peroxide treatment in the G1 and G2 phases is due to the silent repair of oxidative DNA lesions produced at these stages by the base excision repair (BER) pathway. Only the disruption of the BER pathway and the accumulation and/or treatment of DNA intermediates by alternative repair pathways reveal the existence of primary DNA lesions induced at all phases of the cell cycle by hydrogen peroxide. Our data illustrate both the concept of silent repair of DNA damage and the high sensitivity of S-phase cells to hydrogen peroxide. Keywords: budding yeast/checkpoints/DNA damage/oxidative lesions/silent repair

Introduction

Reactive oxygen species (ROS) are a major source of spontaneous damage to DNA, proteins, lipids and carbohydrates. There are various intra- and extracellular sources of oxygen radicals, the major intracellular sources probably being the leakage associated with the reduction of oxygen to water during mitochondrial respiration and the by-products of peroxisomal metabolism. Extracellular sources include ROS generated by macrophages in the inflammatory response and ionizing radiation that produces ROS from the radiolysis of water (Ward, 1988; Riley, 1994).

Oxidative attack on the DNA results in mutagenic structures such as 8-hydroxyadenine and 8-hydroxyguanine (reviewed in Friedberg et al., 1995) and in the instability of repetitive sequences (Jackson et al., 1998), which are all associated with a heightened risk of cancer. Endogenous oxidative damage is extensive, and the level of steady-state oxidative lesions has been estimated at 103–105 adducts per cell in mammals, which is equivalent to or higher than estimates of endogenous non-oxidative adducts (Beckman and Ames, 1997; Helbock et al., 1998). Interestingly, a number of different tumor cell lines were shown to overproduce and accumulate hydrogen peroxide at levels as high as those found in stimulated polymorphonuclear lymphocytes (Szatrowski and Nathan, 1991). This observation has led to the hypothesis that the pro-oxidant state of tumor cells could enhance their neoplastic behavior, and, indeed, progression of human breast cancers to the metastatic state has been linked to hydroxyl radical-induced DNA damage (Malins et al., 1996).

Oxidative stress thus plays an active role in the triggering and progression of many forms of genetic anomaly. In all eukaryotic cells, genome integrity is protected by surveillance mechanisms called DNA checkpoints that, when activated by DNA lesions or replication blocks, induce transcription of DNA repair genes and delay cell cycle progression in order to prevent replication and segregation of damaged DNA molecules (Weinert, 1998). In Saccharomyces cerevisiae, DNA checkpoints inhibit the G1–S transition (Siede et al., 1993, 1994), slow down progression through S phase (Paulovich and Hartwell, 1995) and delay chromosome segregation (Weinert and Hartwell, 1988, 1993; Weinert et al., 1994) when DNA is damaged during the G1, S or G2 phase, respectively. In addition, DNA checkpoints prevent mitosis when DNA replication is blocked.

Proteins encoded by RAD9 and by the genes of the RAD24 epistasis group, including RAD17, MEC3 and DDC1, are required specifically for proper DNA damage response and are proposed to act at an early step of DNA damage recognition (Weinert and Hartwell, 1988; Siede et al., 1993; Weinert et al., 1994; Longhese et al., 1996, 1997; Paulovich et al., 1997; Paciotti et al., 1998, 2000; Vialard et al., 1998; Lowades and Murguia, 2000). In addition, the DNA replication proteins Pol2, Dpb1 and Rfc5 and the Top1 protein appear to sense both replication blocks and DNA damage during S phase (Araki et al., 1995; Navas et al., 1995, 1996; Sugimoto et al., 1996, 1997; Foss, 2001). Signaling by DNA damage or stalled replication forks involves a protein phosphorylation cascade propagated through the essential protein kinases Rad53 and Mec1 (Allen et al., 1994; Weinert et al., 1994; Paulovich and Hartwell, 1995). Mec1 is a member of an evolutionarily conserved family of protein kinases including S.cerevisiae Tel1, Schizosaccharomyces pombe Rad3 and mammalian ATM (ataxia telangiectasia mutated) and ATR (AT-related) (Elledge, 1996). RAD53 is the S.cerevisiae homolog of S.pombe Cds1 and of human hCHK2, which is mutated in some individuals afflicted with an atypical form of the cancer-causing Li–Fraumeni syndrome (Bell et al., 1999). In response to DNA damage or replication blocks, Mec1 is required for the phosphorylation of several substrates, including Rad53, whose phosphorylation state is thus a marker to monitor the activation of the DNA checkpoints.

Although oxidative stress has been implicated in DNA damage and cancer, its effect on DNA checkpoints has...
rarely been examined (Shackelford et al., 2000). We describe here a detailed investigation of the signaling of hydrogen peroxide-induced oxidative stress to the DNA checkpoints of *S. cerevisiae*.

**Results**

**A sublethal oxidative stress promotes the phosphorylation of Rad53**

Endogenous oxidative DNA damage is extensive in aerobic cells, but does not seem to trigger the DNA checkpoints. We investigated whether a sublethal oxidative stress induced by exposure to hydrogen peroxide would affect the DNA surveillance pathways. Rad53 is phosphorylated in response to DNA damage or replication blocks, and its phosphorylation correlates with the activation of the DNA checkpoints (Sanchez et al., 1996; Sun et al., 1996), so we used it as a marker in H2O2-treated cells. Exponentially growing wild-type cells were treated with 0.4 or 0.8 mM H2O2, and Rad53 phosphorylation was monitored in a time course experiment. Rad53 phosphorylation was detected after a 7 min exposure to H2O2 and reached a maximum after 15 min (Figure 1A; data not shown). We investigated the DNA checkpoint activation after 15 min treatments with lower concentrations of H2O2: Rad53 phosphorylation was not observed after treatment with 0.05 or 0.1 mM H2O2 and was weakly induced after treatment with 0.2 mM H2O2 (Figure 1B), suggesting that 0.4 mM H2O2 represents a lower limit for the maximal activation of Rad53 phosphorylation by exposure to H2O2. The H2O2 concentrations used for this study (up to 0.8 mM) are doses that trigger adaptive responses and enhance subsequent oxidative stress resistance (Collinson and Dawes, 1992; Jamieson, 1992) without affecting the cell viability.

**Rad53 phosphorylation in response to low concentrations of hydrogen peroxide is induced specifically in S phase**

We examined Rad53 phosphorylation in wild-type cells synchronized either in G1 by α-factor or in G2-M by nocodazole, and treated with 0.8 mM H2O2 for 15 min. As shown in Figure 2A, Rad53 was not phosphorylated under these conditions, whereas the same treatment triggered Rad53 phosphorylation in exponentially growing cells. Hydrogen peroxide treatment contrasted with UV irradiation, which is able to induce Rad53 phosphorylation in α-factor- and nocodazole-arrested cells (de la Torre-Ruiz et al., 1998; Vialard et al., 1998).

Rad53 phosphorylation in response to H2O2 in asynchronously growing cells, but not in cells blocked in G1 or G2-M, suggested a specific phosphorylation of Rad53 in S-phase cells. We verified this hypothesis by performing a
Rad9, Rad17 and Mec1 are part of the hydrogen peroxide signaling pathways to Rad53 and are necessary to maintain cell viability upon exposure to hydrogen peroxide

MEC1, RAD9 and the members of the RAD24 epistasis group, including RAD17, are required specifically for DNA damage responses (Longhese et al., 1998). We examined the dependence of Rad53 phosphorylation on these genes in exponentially growing mutant cells treated with 0.8 mM H2O2 for 15 min and we found that Rad53 phosphorylation was partially dependent on Rad9 and Rad17, and fully dependent on Mec1 (Figure 3A).

In order to assess the physiological importance of Mec1, Rad53, Rad9 and Rad17 for cell survival upon exposure to H2O2, we compared the viability of wild-type, rad9Δ, rad17Δ, rad9Δ rad17Δ, rad53Δ and mec1Δ cells challenged with increasing concentrations of H2O2. We found that the rad9Δ rad17Δ double mutant and the rad53Δ and mec1Δ single mutants were hypersensitive to H2O2 (Figure 3B), confirming the role of these checkpoint proteins in maintaining cell viability during oxidative stress. Interestingly, the rad9Δ and rad17Δ single mutants were no more sensitive to H2O2 than wild-type cells, whereas they both exhibited a strong sensitivity to UV irradiation (data not shown).

Hydrogen peroxide treatment induces a Rad53-dependent cell cycle delay specifically in S phase

The activation of the DNA checkpoints in response to DNA damage or replication blocks often results in cell cycle delays. We therefore monitored cell cycle progression in wild-type and mutant cells upon exposure to H2O2.

Wild-type cells were synchronized either in G1 by α-factor or in G2–M by nocodazole. The cells subsequently were washed to eliminate the blocking factors and H2O2 was added to the culture medium immediately to a final concentration of 0.8 mM (treatment in G1 or G2, respectively). Alternatively, H2O2 was added to the cell culture 30 min after the release from the α-factor block (treatment in S phase). Hydrogen peroxide treatments induced delays in all phases of the cell cycle (Figures 4 and 5). Treating G1-phase cells with H2O2 resulted in delaying their entry into S phase by ~30 min, as seen by fluorescence-activated cell sorting (FACS) analysis and budding curves (Figure 4A and B). Treatment in the G2–M phase resulted in a 15 min delay, as shown by FACS analysis and the percentage of unbudded cells (Figure 4D and E), whereas H2O2 treatment at the beginning of S phase delayed completion of DNA replication by 45 min (Figure 5A). We verified by western analysis that Rad53 phosphorylation never appeared when the addition of H2O2 took place during G1 or G2 (Figure 4C and F), whereas it was visible 15 min after H2O2 treatment of cells synchronized in S phase and remained so for the next 75 min, even after cells had completed DNA replication (Figure 5, see the 120 min time point). A return to the basal level of Rad53 phosphorylation was observed 120 min after the addition of H2O2 (Figure 5, 150 min time point), which corresponded to the time when cells began to complete mitosis.

The induction of cell cycle delays at all phases upon exposure to H2O2 contrasted with the specific induction of Rad53 phosphorylation during S phase, so we investigated the dependence of the cell cycle delays upon the DNA checkpoints by analysing rad9, rad17, rad53 and mec1 mutants. As shown in Figure 4A and B, the behavior of rad53-21 or rad53Δ cells treated with H2O2 in G1 phase was similar to that of wild-type cells, since they delayed their entry into S phase by ~30 min compared with untreated cells. A similar delay in G1 was also observed in rad9Δ, rad17Δ and mec1Δ cells after H2O2 treatment (data not shown), demonstrating that this cell cycle response was independent of the DNA checkpoint pathways. Similarly, H2O2 treatment of rad53-21, rad53Δ and rad9Δ mutant cells synchronized in the G2–M phase resulted in a 15 min delay identical to that of wild-type cells (Figure 4D and E, and data not shown), indicating that the G2–M delay induced by H2O2 treatment was not controlled by the DNA checkpoints.
Finally, we investigated whether the slowing of DNA replication induced by H$_2$O$_2$ treatment of S-phase cells was dependent upon the DNA checkpoints. $rad9^\Delta$ $rad17^\Delta$, $rad53^\Delta$, $rad53$ and $mec1^\Delta$ mutant cells released from the blocking factors and immediately treated with 0.8 mM H$_2$O$_2$ or left untreated. (A) FACS analysis of cells synchronized in G$_1$ and either treated with 0.8 mM H$_2$O$_2$ (+) or left untreated (−). Aliquots were taken from exponentially growing cells (exp), α-factor-synchronized cells (αF) and at the indicated times after release from α-factor. (B) Percentage of budded cells in α-factor-synchronized wild-type or $rad53^\Delta$ cell cultures either treated with 0.8 mM H$_2$O$_2$ at $t_0$ min (closed symbols) or left untreated (open symbols), at the indicated times after α-factor release. (C) Western blot analysis of total proteins from α-factor-synchronized wild-type cells either treated with 0.8 mM H$_2$O$_2$ or left untreated, at the indicated times after release from α-factor. (D) FACS analysis of cells synchronized in G$_2$–M phase and either treated with 0.8 mM H$_2$O$_2$ (+) or left untreated (−). Aliquots were taken from exponentially growing cells (exp), nocodazole-synchronized cells (noc) and at the indicated times after release from nocodazole block. (E) Percentage of unbudded cells in nocodazole-synchronized wild-type or $rad53^\Delta$ cell cultures either treated with 0.8 mM H$_2$O$_2$ at $t_0$ min (closed symbols) or left untreated (open symbols), at the indicated times after release from nocodazole block. (F) Western blot analysis of total proteins from nocodazole-synchronized wild-type cells either treated with 0.8 mM H$_2$O$_2$ or left untreated, at the indicated times after release from nocodazole block.

Finally, we investigated whether the slowing of DNA replication induced by H$_2$O$_2$ treatment of S-phase cells was dependent upon the DNA checkpoints. $rad9^\Delta$ $rad17^\Delta$, $rad53^\Delta$, $rad53^\Delta$ and $mec1^\Delta$ mutant cells released from α-factor block were treated with H$_2$O$_2$ at the beginning of S phase, and DNA replication was monitored by FACS analysis. However, untreated $mec1^\Delta$ and $rad53^\Delta$ cells had a slow and poorly synchronized S phase, in keeping with a postulated role for these kinases in stimulating DNA replication (Desany et al., 1998), which made it difficult to draw conclusions concerning the effect of adding H$_2$O$_2$ (Figure 5A and data not shown). In contrast, the checkpoint-deficient allele $rad53^\Delta$-$21$ allows an efficient DNA replication. Hydrogen peroxide-treated and untreated $rad53^\Delta$-$21$ cells were found to proceed at a similar pace through S phase (Figure 5A), indicating that the slowing of DNA replication in response to H$_2$O$_2$ treatment was under control of the Rad53 pathways. The behavior of $rad53^\Delta$-$21$ cells contrasted with that of $rad9^\Delta$ $rad17^\Delta$ cells, whose completion of DNA replication after H$_2$O$_2$ treatment was delayed to the same extent as in wild-type cells (Figure 5A). The specific dependence on Rad53 of the cell cycle delay induced by H$_2$O$_2$ in S phase was thus consistent with the specific induction of Rad53 phosphorylation in that phase. The fact that the delay in DNA replication was independent of Rad9 and Rad17 was in keeping with the fact that Rad53 was still partially phosphorylated in the $rad9^\Delta$ $rad17^\Delta$ mutant after H$_2$O$_2$ treatment.

Incomplete processing of primary oxidative DNA lesions by the BER pathway reveals the presence of DNA damage induced by hydrogen peroxide at all phases of the cell cycle

The lack of Rad53 phosphorylation after H$_2$O$_2$ treatment in G$_1$ and G$_2$ could be explained by two different
hypotheses: DNA lesions could be induced by H$_2$O$_2$ exclusively during S phase, due to an enhanced sensitivity of replicating DNA, or DNA damage could appear at all phases of the cell cycle, but would be signaled to Rad53 only during S phase. In order to gain some insight into the molecular events triggered by H$_2$O$_2$, we analyzed mutant cells affected in DNA repair pathways.

Oxidative DNA damage is thought to be processed primarily by the base excision repair (BER) pathway (Friedberg et al., 1995). The nucleotide excision repair (NER) pathway appears to play a secondary role in the repair of these lesions, whereas recombination and translesion synthesis occur as mechanisms of damage tolerance (Swanson et al., 1999). Hydroxyl radicals, the main active derivatives of H$_2$O$_2$, affect DNA either by oxidizing its bases or by generating single strand breaks. Oxidized bases are processed through the action of BER DNA glycosylases/AP lyases into abasic sites with a single strand break. These intermediates must then be processed further by the AP endonucleases Apn1 or Apn2 before DNA polymerases and ligases of the BER pathway can complete the repair (Friedberg et al., 1995). We reasoned that by disrupting the APN1 and APN2 genes, we would interrupt the processing of primary oxidative lesions, forcing either the accumulation of intermediate adducts or their treatment by alternative DNA repair pathways that might signal to Rad53.

apn1Δ apn2Δ mutant cells were synchronized either in G$_1$ by α-factor or in G$_2$–M by nocodazole and treated with 0.8 mM H$_2$O$_2$ for 15 min in the presence of the blocking factors. As shown in Figure 6A, this treatment triggered a strong Rad53 phosphorylation in the apn1Δ apn2Δ cells arrested in G$_1$ or in G$_2$–M, whereas, as already discussed, Rad53 was not modified under these conditions in wild-type cells. A time course experiment similar to that presented in Figure 2B, in which synchronized cells were treated with 0.8 mM H$_2$O$_2$ for 15 min at different times after release from α-factor block, confirmed that H$_2$O$_2$ induced Rad53 phosphorylation in apn1Δ apn2Δ cells at all phases of the cell cycle, and showed that during S phase the relative amount of phosphorylated forms of Rad53 was higher in apn1Δ apn2Δ than in wild-type cells (Figure 6B and C).

In order to confirm that the phosphorylation of Rad53 observed in G$_1$- or G$_2$-synchronized apn1Δ apn2Δ cells indeed reflected the activation of the DNA checkpoints, we analyzed the cell cycle progression of apn1Δ apn2Δ and apn1Δ apn2Δ rad53Δ cells after H$_2$O$_2$ treatment. Exposure of α-factor- or nocodazole-synchronized apn1Δ apn2Δ cells to 0.8 mM H$_2$O$_2$ resulted in delays of 60 and >90 min, respectively, as determined by FACS analysis and budding curves (Figure 7). These delays were much longer than those observed for wild-type cells, which amounted respectively to 30 and 15 min. The delay observed after H$_2$O$_2$ treatment of nocodazole-synchronized apn1Δ apn2Δ rad53Δ cells was reduced strikingly to some 30 min according to DNA content and budding index analyses, demonstrating that most of the extra delay induced in G$_2$–M-phase apn1Δ apn2Δ cells was controlled by Rad53 (Figure 7C and D). Similarly, treating α-factor-synchronized apn1Δ apn2Δ rad53Δ cells with 0.8 mM H$_2$O$_2$ resulted

Fig. 5. Hydrogen peroxide induces a Rad53-dependent cell cycle delay specifically in S phase. Exponentially growing wild-type (MCM185), rad9Δ rad17Δ (MCM247), rad53Δ (MCM196) and rad53-21 (MCM257) cells were synchronized in G$_1$ with α-factor and treated with 0.8 mM H$_2$O$_2$ 30 min after the release from the α-factor block or left untreated. (A) FACS analysis of cells either treated with 0.8 mM H$_2$O$_2$ (+) or left untreated (−). Aliquots were taken from exponentially growing cells (exp), α-factor-synchronized cells (αF) and at the indicated times after release from α-factor block. (B) Western blot analysis of total proteins from α-factor-synchronized wild-type cells either treated with 0.8 mM H$_2$O$_2$ or left untreated, at the indicated times after release from α-factor block.
in delaying their entry into S phase by only 30 min, as seen by the budding curves (Figure 7B). The reduction of this delay could not be observed with the FACS data (Figure 7A), probably because the absence of Apn1 and Apn2 dramatically increased radical-induced DNA lesions, some of which have been demonstrated to block DNA polymerases (Friedberg et al., 1995). Despite the lack of DNA checkpoints, DNA replication thus would be mechanically stalled in apn1Δ apn2Δ cells after H2O2 treatment, whereas the budding process remained unaffected and released the Rad53 dependence of the extra delay observed after exposure to H2O2 of G1-phase apn1Δ apn2Δ cells. Rad53 phosphorylation observed in G1 and in G2-M apn1Δ apn2Δ cells was thus correlated with Rad53-dependent extra delays in cell cycle progression, i.e. activation of the DNA checkpoints.

Since the absence of Apn1 and Apn2 can only affect the processing and not the formation of DNA lesions, we concluded that (i) DNA lesions are produced in G1, S and G2 phases upon treatment with 0.8 mM H2O2; (ii) these lesions do not induce the phosphorylation of Rad53 when they are normally processed by the BER pathway; and (iii) they trigger Rad53 phosphorylation when they cannot be processed by Apn1 and Apn2.

Siede et al. (1994) and Neecke et al. (1999) have shown that the induction of cell cycle delay and of Rad53 phosphorylation in response to UV-induced DNA damage in G1 phase is dependent upon the presence of Rad14, a protein involved in the early steps of the NER pathway. These results demonstrated that the UV-induced DNA lesions by themselves were unable to trigger the phosphorylation of Rad53 and the activation of the DNA checkpoints, and that only their processing by the NER pathway could elicit a signal to the DNA checkpoints. Although oxidative DNA lesions are removed predominantly by the BER pathway, Swanson et al. (1999) have recently shown that the NER pathway could also contribute to their processing, and we observed that the sensitivity to H2O2 of wild-type and apn1Δ apn2Δ cells was enhanced by the disruption of RAD14 (Figure 6D). We therefore investigated whether in apn1Δ apn2Δ cells the processing by the NER pathway of either the primary oxidative DNA lesions or their derivatives was responsible for triggering the phosphorylation of Rad53. However,
Rad53 phosphorylation was found to be similar in **apn1Δ apn2Δ** and in **apn1Δ apn2Δ rad53Δ** cells at all phases of the cell cycle (Figure 6A–C), indicating that Rad14 was not required for this signaling.

**Discussion**

ROS have been implicated in the appearance and evolution of genetic anomalies, yet few studies have addressed their effects on the cell cycle and the DNA checkpoints. Here we show that exposure to low concentrations of H$_2$O$_2$ delays cell cycle progression in G$_1$, S and G$_2$ phases, but that only the delay occurring in S phase is controlled by the DNA checkpoints. Consistent with this observation, we found that Rad53 phosphorylation is induced by H$_2$O$_2$ treatment specifically during S phase. Finally, we demonstrated that the lack of Rad53 phosphorylation after H$_2$O$_2$ treatment in the G$_1$ and G$_2$ phases is due to the silent repair by the BER pathway of oxidative DNA lesions produced at these stages.

**Sublethal oxidative stresses are sensed by the DNA surveillance pathways**

Sublethal oxidative stresses induced by low concentrations of H$_2$O$_2$ are detected by the DNA checkpoint pathways in yeast and trigger Rad53-dependent responses. These observations enrich our understanding of cellular responses to oxidative stress and could have important implications for other systems. The human hereditary diseases ataxia telangiectasia, Fanconi’s anemia and Bloom’s syndrome, characterized by increased cancer incidence, are also linked to abnormalities in oxygen metabolism (Rotman and Shiloh, 1997; for a review see Cerutti, 1985). Moreover, numerous types of tumor cell
lines were shown to accumulate high levels of H$_2$O$_2$ (Sztarowski and Nathan, 1991). Conceivably, pro-oxidant states or increased levels of permanent oxidative stress leading to continuous checkpoint activation could induce some form of adaptation and result in the defective functioning of these pathways, eliciting abnormal responses to genotoxic insults and favoring uncontrolled cell proliferation.

**Hydrogen peroxide-induced delays in G$_1$ and in G$_2$ are independent of the DNA checkpoints**

We have demonstrated that only the delay caused in S phase by low concentrations of H$_2$O$_2$ is dependent upon Rad53. The mechanisms responsible for the delays occurring in the G$_1$ and G$_2$ phases after H$_2$O$_2$ treatment remain unknown but they could be triggered by cellular reactions other than oxidative attacks on DNA. Several non-genotoxic agents have been reported to cause delays in G$_1$. When glucose is added to cells growing in a poor carbon source, the critical cell size required for Start is reset from a small to a large size through glucose stimulation of the Ras/cAMP pathway, which represses expression of *CLN1* (Flick *et al.*, 1998). Likewise, heat shock causes a transient inhibition of Start through decreasing *CLN1* and *CLN2* transcripts (Rowley *et al.*, 1993). Regarding oxidative stress, a sod1A mutant (affected in the cytosolic Cu, Zn-superoxide dismutase) exposed to 100% O$_2$ was found to arrest permanently in G$_1$ through an inhibition of *CLN1* and *CLN2* transcription (Lee *et al.*, 1996), but the involvement of the DNA checkpoints in this inhibition was not investigated. A similar mechanism may operate in wild-type cells treated with H$_2$O$_2$ in G$_1$. However, the regulatory pathways responsible for the G$_1$ and G$_2$–M delays in wild-type cells exposed to oxidative stress remain to be determined.

**Hydrogen peroxide treatment triggers Rad53 phosphorylation specifically during S phase**

The induction of Rad53 phosphorylation specifically after addition of H$_2$O$_2$ to S-phase cells could be explained by an effect of low concentrations of H$_2$O$_2$ on ribonucleotide reductase (RNR) activity through depletion of reducing agents. However, Rad53 phosphorylation in S phase upon exposure to H$_2$O$_2$ is more intense in *apn1A* *apn2A* cells than in wild-type cells, which strongly suggests that DNA lesions are induced by H$_2$O$_2$ in this phase. Furthermore, the observation that Rad53 phosphorylation in wild-type cells treated with H$_2$O$_2$ is partially dependent on Rad9 and Rad17, whereas Rad53 phosphorylation triggered by the inactivation of the RNR is completely independent of Rad9 and Rad17 (Pellicioli *et al.*, 1999; our unpublished data) is consistent with the presence of DNA lesions in S phase. Several hypotheses could account for the specific induction of Rad53 phosphorylation in this phase. First, besides inducing DNA damage, H$_2$O$_2$ treatment could also affect RNR activity, trigger a shortage of dNTP and generate a signal to Rad53 that could synergistically enhance a weak signal induced by the processing of DNA lesions. A second hypothesis may be that H$_2$O$_2$ induces more or different DNA damage during S phase because of the intrinsic sensitivity of replicating DNA, or because DNA replication converts primary lesions into DNA structures (DNA breaks, single-stranded DNA or recombination intermediates generated by the stalling of the replication fork) recognizable by DNA damage sensors (Foriani *et al.*, 1998). Thirdly, mechanisms capable of sensing H$_2$O$_2$-induced DNA damage could operate in S phase but not in G$_1$ or G$_2$. The DNA replication proteins Pol2, Dpb11 and Rfc5 and the Tof1 protein appear to sense both replication blocks and DNA damage specifically during DNA synthesis (Araki *et al.*, 1995; Navas *et al.*, 1995, 1996; Sugimoto *et al.*, 1996, 1997; Foss, 2001) and could be involved in the signaling of H$_2$O$_2$-induced DNA lesions to Rad53.

**DNA lesions induced in G$_1$ and G$_2$ by low concentrations of hydrogen peroxide are repaired silently by the BER pathway in wild-type cells and only trigger Rad53 phosphorylation when they are processed incompletely by this pathway**

A link between the treatment of UV-induced DNA lesions and the activation of the DNA checkpoints was demonstrated recently in yeast by Sieide *et al.* (1994) and Neecke *et al.* (1999), who showed that the UV-induced activation of the Rad53 checkpoint pathway in G$_1$ is strictly dependent on the NER protein Rad14. Similarly, XP-A lymphoblasts (affected in the human homolog of *RAD14*) require much higher doses of UV irradiation than wild-type cells in order to induce p53 expression when DNA replication is inhibited (Nelson and Kastan, 1994). These studies showed that DNA lesions induced by low doses of UV irradiation do not constitute *per se* a sufficient signal for DNA checkpoint activation in G$_1$ or when DNA replication is inhibited, and that only the presence of NER complexes or the appearance of DNA intermediates derived from their processing of the primary DNA lesions are able to activate the DNA surveillance pathways.

In contrast to UV-induced DNA damage, DNA lesions produced by low concentrations of H$_2$O$_2$ in G$_1$ or G$_2$ are able to activate the DNA checkpoints only when they are processed incompletely by the BER pathway. In wild-type cells, DNA lesions induced by H$_2$O$_2$ treatment in G$_1$ or G$_2$ are repaired silently by Apn1/Apn2-dependent pathways, in the sense that their processing does not activate the DNA checkpoints. The repair of DNA lesions thus is not linked systematically to the activation of the DNA checkpoints and does not appear to require it for proper efficiency. We found that DNA lesions induced in G$_1$ cells by 0.8 mM H$_2$O$_2$ were unable to trigger Rad53 phosphorylation when the cells subsequently reached the S phase (Figure 4A and C), which demonstrated that the lesions were repaired in G$_1$ to such an extent as not to constitute a sufficient signal for the checkpoints in S phase.

The various types of DNA lesions and their respective repair systems thus can be classified by their ability to trigger the activation of the DNA surveillance pathways in the absence of DNA replication. Processing by the NER, Rad14-dependent pathway of UV-induced DNA lesions seems easily detected and signaled to Rad53, whereas processing of H$_2$O$_2$-induced lesions by the BER, Apn1/Apn2-dependent pathway seems to go unnoticed by the DNA checkpoints. In the absence of Apn1 and Apn2, primary oxidative lesions are converted by glycosylases/ AP-lyases into abasic sites with a single strand break. What then induces Rad53 phosphorylation? Apn1/Apn2 substrates could be either sensed directly by the check-
Fig. 8. Comparison of the processing and signaling of UV- or H₂O₂-induced DNA lesions during the G₁ phase. The treatment of UV-induced DNA damage by Rad14-dependent pathways (NER) allows both its repair and its signaling to Rad53. In contrast, the processing of H₂O₂-induced DNA damage by Apn1/2-dependent pathways (BER) results in its repair and the absence of DNA checkpoint activation.

Materials and methods

Yeast strains

Strains used in this study are listed in Table I. All yeast strains are isogenic to MCM185 (MATa, ura3-52, lys2-801, ade2-101, trplΔ::LEU2, his3Δ200, leu2Δ1, bar1Δ::LEU2), a YPH499 derivative in which the BARR gene has been inactivated by the insertion of a LEU2 cassette (MacKay et al., 1988; Sikorski and Hieter, 1989). To construct RAD9, RAD17, RAD53, APN1, APN2 and RAD14 chromosomal deletions, the radΔ::kanMX4, rad9Δ::URA3, rad17Δ::kanMX4, rad53Δ::kanMX4, apn1Δ::kanMX4, apn2Δ::URA3 and rad14Δ::URA3 cassettes were produced by PCR using either plasmid pFA6a-kanMX4 (Wach et al., 1994) or the Kluyveromyces lactis URA3 gene as a template and oligonucleotides RAD9D5 (5'-TAGAAAAAGCATATGGAAGAAAATTCTTCAACATCGGCGCTGCG-3') and RAD9D3 (5'-ATTGATTACGTATTTAAATTCTTCTCGCTTCA-3') as primers. These observations point to additional roles for the Rad1–Rad10 complex in DNA repair pathways that would be linked to Apn1 but distinct from the NER activity. Furthermore, the deletion of RAD1 was found to be colchetal with the deletion of APN1 and APN2 (M. Guillet and S. Boiteux, personal communication), whereas the triple apn1Δ apn2Δ rad14Δ mutant does not exhibit any growth defects. This observation also suggests that Rad1, an endonuclease involved in many repair pathways, could represent an alternative enzyme for the treatment of Apn1/2 substrates and, potentially, their signaling to the DNA checkpoints.

Altogether, our data illustrate the concept of silent repair of DNA damage and demonstrate the high sensitivity of S phase cells to H₂O₂. Given the importance of oxidative DNA lesions in aerobic cells, it will be interesting to determine the identity and activity of oxidative DNA damage sensors in yeast as well as in mammalian cells.

Materials and methods

Yeast strains

Strains used in this study are listed in Table I. All yeast strains are isogenic to MCM185 (MATa, ura3-52, lys2-801, ade2-101, trplΔ::LEU2, his3Δ200, leu2Δ1, bar1Δ::LEU2), a YPH499 derivative in which the BARR gene has been inactivated by the insertion of a LEU2 cassette (MacKay et al., 1988; Sikorski and Hieter, 1989). To construct RAD9, RAD17, RAD53, APN1, APN2 and RAD14 chromosomal deletions, the radΔ::kanMX4, rad9Δ::URA3, rad17Δ::kanMX4, rad53Δ::kanMX4, apn1Δ::kanMX4, apn2Δ::URA3 and rad14Δ::URA3 cassettes were produced by PCR using either plasmid pFA6a-kanMX4 (Wach et al., 1994) or the Kluyveromyces lactis URA3 gene as a template and oligonucleotides RAD9D5 (5'-TAGAAAAAGCATATGGAAGAAAATTCTTCAACATCGGCGCTGCG-3') and RAD9D3 (5'-ATTGATTACGTATTTAAATTCTTCTCGCTTCA-3') as primers. These observations point to additional roles for the Rad1–Rad10 complex in DNA repair pathways that would be linked to Apn1 but distinct from the NER activity. Furthermore, the deletion of RAD1 was found to be colchetal with the deletion of APN1 and APN2 (M. Guillet and S. Boiteux, personal communication), whereas the triple apn1Δ apn2Δ rad14Δ mutant does not exhibit any growth defects. This observation also suggests that Rad1, an endonuclease involved in many repair pathways, could represent an alternative enzyme for the treatment of Apn1/2 substrates and, potentially, their signaling to the DNA checkpoints.

Altogether, our data illustrate the concept of silent repair of DNA damage and demonstrate the high sensitivity of S phase cells to H₂O₂. Given the importance of oxidative DNA lesions in aerobic cells, it will be interesting to determine the identity and activity of oxidative DNA damage sensors in yeast as well as in mammalian cells.
Table 1. Saccharomyces cerevisiae strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>YPH499</td>
<td>MATA, ura3-52, lys2-801amber, ade2-101oche, trpl−A63, his3Δ200, leu2−1Δ</td>
<td>Sikorski and Hieter (1989)</td>
</tr>
<tr>
<td>MCM185</td>
<td>as YPH499 bar1Δ−LEU2</td>
<td>this study</td>
</tr>
<tr>
<td>MCM188</td>
<td>as MCM185 rad17Δ::kanMX4</td>
<td>this study</td>
</tr>
<tr>
<td>MCM189</td>
<td>as MCM185 mec1Δ::HIS3 + pBAD70 (2μ, TRP1, GAP-RNR1)</td>
<td>this study</td>
</tr>
<tr>
<td>MCM196</td>
<td>as MCM185 rad53Δ::kanMX4 + pBAD70 (2μ, TRP1, GAP-RNR1)</td>
<td>this study</td>
</tr>
<tr>
<td>MCM215</td>
<td>as MCM185 rad9Δ::kanMX4</td>
<td>this study</td>
</tr>
<tr>
<td>MCM247</td>
<td>as MCM185 rad17Δ::kanMX4 rad9Δ::URA3</td>
<td>this study</td>
</tr>
<tr>
<td>MCM258</td>
<td>as MCM185 rad53Δ::kanMX4 + pBAD70 (2μ, TRP1, GAP-RNR1) + pJA92/rad53−21</td>
<td>this study</td>
</tr>
<tr>
<td>MCM279</td>
<td>as MCM185 apn1Δ::kanMX4</td>
<td>this study</td>
</tr>
<tr>
<td>MCM305</td>
<td>as MCM185 rad14Δ::URA3</td>
<td>this study</td>
</tr>
<tr>
<td>MCM309</td>
<td>as MCM185 apn1Δ::kanMX4 apn2Δ::ura3 rad14Δ::URA3</td>
<td>this study</td>
</tr>
<tr>
<td>MCM332</td>
<td>as MCM185 apn1Δ::kanMX4 apn2Δ::URA3 rad53Δ::kanMX4 + pBAD70 (2μ, TRP1, GAP-RNR1)</td>
<td>this study</td>
</tr>
</tbody>
</table>

Acknowledgements

We wish to thank Michel Toledano, Stéphane Marcaud, Jean Labarde and Serge Boiteux for useful discussions. This work was financed in part by a specific radiobiology action grant from the Ministère de l’Éducation Nationale, de la Recherche et de la Technologie. C.L. was supported by an EDF/CEA fellowship.

References


give rise to apn1Δ apn2Δ rad14Δ triple mutant MCM309. The rad9Δ rad17Δ mutant MCM247 was generated by transforming the rad17Δ strain MCM188 by the rad9Δ::URA3 PCR product. To construct strain MCM189, carrying the *MEC1* chromosomal deletion, MCM185 was transformed with the *EcoRI*-linearized plasmid sp309 (a gift from S. Marcand, CEA/Saclay, France), which harbors a *meclΔ::HIS3* cassette cloned into the *KpnI* site of plasmid pUC18. As RAD53 and *MEC1* are essential genes, strain MCM185 was transformed with pBAD70 (Desany et al., 1998), a multicopy vector that carries the *GAP-RNR1* construct, a suppressor of rad53Δ and mec1Δ lethality, prior to the construction of RAD53 and *MEC1* chromosomal deletions. The accuracy of the RAD9, *RAD17*, RAD53, *APN1*, *APN2* and *RAD14* gene replacements was verified by PCR analysis of genomic DNA. Strain MCM257 was constructed by introducing *pJA92/rad53−21*, a centromeric plasmid harboring the *rad53Δ−21* (also known as *sad1−1*) allele, into MCM196. *pJA92/rad53−21* was created as follows: *pJA92*, a URA3-marked plasmid bearing a wild-type copy of *RAD53* (Allen et al., 1994), was digested by *AflII* and *SacI*, whose sites are located on either side of the *RAD53* reading frame, and the purified fragment containing only the 5′ and the 3′ borders of *RAD53* was introduced into Y301, a strain harboring the *rad53Δ−21* allele (Allen et al., 1994). *Ura*+ transformants were recovered and the *URA3* marked plasmids extracted. One of these plasmids, *pJA92/rad53−21*, exhibited a restriction pattern similar to *pJA92* and, when introduced into MCM196, conferred resistance to 5 mM hydroxyurea (HU), but not to 20 mM HU, which corresponds to the Y301 phenotype. The apn1Δ apn2Δ rad53Δ::kanMX4 mutant MCM32 was generated by crossing MCM196 and MCM283 after changing MCM283 mating type; *Ura*+, *Trp*+, *Leu*+, *G418* resistant and HU-sensitive spores were selected and the deletion of the genes was tested further by PCR analysis.

Cells grown at 30°C in YPD medium (1% yeast extract, 2% bactopeptone, 2% glucose). Transformants carrying the *rad9Δ::kanMX4, rad17Δ::kanMX4, rad53Δ::kanMX4* and *apn1Δ::kanMX4* cassettes were selected on YPD plates containing 200 μg/ml G418.

**Synchronization and DNA damage treatments**

Cells grown to log phase in YPD medium were blocked in G1 by the addition of 1 μM α-factor for 2.5 h or in G2 by the addition of 15 μg/ml nocodazole for 3 h. For hydrogen peroxide treatments, hydrogen peroxide was added directly to the cell medium to a final concentration of 0.4–8 mM. To assess cell viability after treatment, the cells were plated onto YPD medium and allowed to grow for another 24 h. Cell viability was then determined by microscopic observation. UV irradiation was performed using a Stratalinker 1800 (Stratagene).

**Analysis of Rad53 phosphorylation**

Analysis of Rad53 phosphorylation was performed as described previously in Marsolier et al. (2000).


Received November 28, 2000; revised March 15, 2001; accepted April 3, 2001