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 10⁴–10⁵ 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; Lowndes and Murguia, 2000). In addition, the DNA replication proteins Pol2, Dpb1 and Rfc5 and the Tof1 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

Fig. 1. Sublethal oxidative stresses promote Rad53 phosphorylation. (A) Western blot analysis of total proteins from exponentially growing wild-type cells (MCM185) treated with 0.4 mM H_2O_2 . Aliquots were taken from untreated cells (exp) and from cells treated with H_2O_2 for the indicated times (min). (B) Western blot analysis of total proteins from exponentially growing wild-type cells (MCM185) treated for 15 min with 0.05, 0.1, 0.2 or 0.4 mM H_2O_2 .

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 H_2O_2 -treated cells. Exponentially growing wild-type cells were treated with 0.4 or 0.8 mM H_2O_2 , and Rad53 phosphorylation was monitored in a time course experiment. Rad53 phosphorylation was detected after a 7 min exposure to H_2O_2 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 H_2O_2 . Rad53 phosphorylation was not observed after treatment with 0.05 or 0.1 mM H_2O_2 and was weakly induced after treatment with 0.2 mM H_2O_2 (Figure 1B), suggesting that 0.4 mM H_2O_2 represents a lower limit for the maximal activation of Rad53 phosphorylation by exposure to H_2O_2 . The H_2O_2 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 G_1 by α -factor or in G_2 -M by nocodazole, and treated with 0.8 mM H_2O_2 for 15 min. As shown in Figure 2A, Rad53 was not phosphorylated under

Fig. 2. Rad53 phosphorylation in response to hydrogen peroxide is induced specifically in S phase. (A) Western blot analysis of total proteins from exponentially growing, G_1 - or G_2 -M-arrested wild-type cells subjected to different genotoxic stresses. Exponentially growing wild-type (MCM185) cell cultures were divided into three equal aliquots. One aliquot was left untreated (exp), and the other two were treated either with α -factor (α F) or with nocodazole (noc). Exponentially growing, G_1 -arrested and G_2 -M-arrested cells subsequently were either left untreated (lane 1), treated with 0.16 M hydroxyurea (HU) for 1 h (lane 2), treated with 0.8 mM H_2O_2 for 15 min (lane 3) or UV irradiated at 80 J/m² and harvested 30 min later (lane 4). The G_1 - and G_2 -M-arrested cells were maintained in their respective phases until harvest by the continuous presence of α -factor and nocodazole, respectively. (B and C) Exponentially growing wild-type cells (MCM185) were synchronized in G_1 with α -factor, released from the pheromone block and treated for 15 min with 0.8 mM H_2O_2 at different times after release. (B) Western blot analysis of total proteins from treated cells and untreated controls. (C) FACS analysis showing the relative DNA content of the synchronized cells after the 15 min H_2O_2 treatment when aliquots were collected for western analysis. In both cases, the indicated time corresponds to the start of the treatment. Untreated exponentially growing (exp) and G_1 -arrested (α F) controls are also shown.

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 H_2O_2 in asynchronously growing cells, but not in cells blocked in G_1 or G_2 -M, suggested a specific phosphorylation of Rad53 in S-phase cells. We verified this hypothesis by performing a

time course experiment. Wild-type cells were synchronized in G₁ with α -factor, released from the pheromone block and treated for 15 min with 0.8 mM H₂O₂ at different times after release. Rad53 phosphorylation became visible in cells treated 15 min after α -factor release, but was mostly apparent when cells were treated 30 or 45 min after release (Figure 2B), at which time they were predominantly in S phase (Figure 2C). Rad53 phosphorylation was strongly attenuated when cells were treated 60 min after α -factor release, when most cells had completed DNA replication.

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

Fig. 3. 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. (A) Exponentially growing wild-type (MCM185) and *rad9Δ* (MCM215), *rad17Δ* (MCM188) *rad9Δ rad17Δ* (MCM247) or *mec1Δ* (MCM189) mutant cells were either left untreated or were treated with 0.8 mM H₂O₂ for 15 min. Protein extracts were analysed by western blots. (B) H₂O₂ sensitivity of different mutants affected in the DNA checkpoint pathways. Exponentially growing wild-type (MCM185) and *rad9Δ* (MCM215), *rad17Δ* (MCM188), *rad9Δ rad17Δ* (MCM247), *rad53Δ* (MCM196) or *mec1Δ* (MCM189) mutant cells were treated in YPD liquid medium with 0, 2, 4 or 8 mM H₂O₂ for 2 h. Samples subsequently were diluted and plated on YPD solid medium to assess cell viability.

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.

Fig. 4. Hydrogen peroxide-induced delays in G_1 and in G_2 are independent of the DNA checkpoints. Exponentially growing wild-type (MCM185), *rad53Δ* (MCM196) and *rad53-21* (MCM257) cells were synchronized in G_1 with α -factor (A, B and C), or in G_2 -M with nocodazole (D, E and F), released from the blocking factors and immediately treated with 0.8 mM H_2O_2 or left untreated. (A) FACS analysis of cells synchronized in G_1 and either treated with 0.8 mM H_2O_2 (+) or left untreated (–). Aliquots were taken from exponentially growing cells (exp), α -factor-synchronized cells (α F) and at the indicated times after release from α -factor. (B) Percentage of budded cells in α -factor-synchronized wild-type or *rad53-21* cell cultures either treated with 0.8 mM H_2O_2 at $t = 0$ min (closed symbols) or left untreated (open symbols), at the indicated times after release from α -factor. (C) Western blot analysis of total proteins from α -factor-synchronized wild-type cells either treated with 0.8 mM H_2O_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_2O_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 *rad53-21* cell cultures either treated with 0.8 mM H_2O_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_2O_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_2O_2 treatment of S-phase cells was dependent upon the DNA checkpoints. *rad9Δ rad17Δ*, *rad53-21*, *rad53Δ* and *mec1Δ* mutant cells released from α -factor block were treated with H_2O_2 at the beginning of S phase, and DNA replication was monitored by FACS analysis. However, untreated *mec1Δ* and *rad53Δ* 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_2O_2 (Figure 5A and data not shown). In contrast, the checkpoint-deficient allele *rad53-21* allows an efficient DNA replication. Hydrogen peroxide-treated and untreated *rad53-21* 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_2O_2 treatment was under control of the Rad53 pathways. The behavior of *rad53-21*

cells contrasted with that of *rad9Δ rad17Δ* cells, whose completion of DNA replication after H_2O_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_2O_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 *rad9Δ rad17Δ* mutant after H_2O_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_2O_2 treatment in G_1 and G_2 could be explained by two different

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 G₁ with α -factor and treated with 0.8 mM H₂O₂ 30 min after the release from the α -factor block or left untreated. (A) FACS analysis of cells either treated with 0.8 mM H₂O₂ (+) or left untreated (–). Aliquots were taken from exponentially growing cells (exp), α -factor-synchronized cells (α F) 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 H₂O₂ or left untreated, at the indicated times after release from α -factor block.

hypotheses: DNA lesions could be induced by H₂O₂ 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 H₂O₂, 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 H₂O₂, 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 G₁ by α -factor or in G₂–M by nocodazole and treated with 0.8 mM H₂O₂ 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 G₁ or in G₂–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 H₂O₂ for 15 min at different times after release from α -factor block, confirmed that H₂O₂ 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 G₁- or G₂-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 H₂O₂ treatment. Exposure of α -factor- or nocodazole-synchronized *apn1Δ apn2Δ* cells to 0.8 mM H₂O₂ 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 H₂O₂ 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 G₂–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 H₂O₂ resulted

Fig. 6. Hydrogen peroxide treatment triggers Rad53 phosphorylation at all phases of the cell cycle in *apn1Δ apn2Δ* cells. (A) Western blot analysis of total proteins from G_1 - or G_2 -M-arrested cells treated with 0.8 mM H_2O_2 . Exponentially growing wild-type (MCM185), *rad14Δ* (MCM305), *apn1Δ apn2Δ* (MCM283) and *apn1Δ apn2Δ rad14Δ* (MCM309) cells were synchronized in G_1 with α -factor (αF) or in G_2 -M with nocodazole (noc), and either left untreated (-) or treated with 0.8 mM H_2O_2 (+) for 15 min in the presence of the blocking factors. (B and C) Exponentially growing wild-type (MCM185), *rad14Δ* (MCM305), *apn1Δ apn2Δ* (MCM283) and *apn1Δ apn2Δ rad14Δ* (MCM309) cells were synchronized in G_1 with α -factor, released from the pheromone block and treated for 15 min with 0.8 mM H_2O_2 at different times after release. (B) Western blot analysis of total proteins from treated cells and untreated controls. (C) FACS analysis showing the relative DNA content of the synchronized cells after the 15 min H_2O_2 treatment when aliquots were collected for western analysis. In both cases, the indicated time corresponds to the start of the treatment. Untreated exponentially growing (exp) and G_1 -arrested (αF) controls are also shown. (D) H_2O_2 sensitivity of various mutants affected in the BER or the NER pathways. Exponentially growing wild-type (MCM185), *rad14Δ* (MCM305), *apn1Δ apn2Δ* (MCM283) and *apn1Δ apn2Δ rad14Δ* (MCM309) cells were treated in YPD liquid medium with 0, 2, 4 or 8 mM H_2O_2 for 2 h. Samples subsequently were diluted and plated on YPD solid medium to assess cell viability.

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 rad53Δ* cells after H_2O_2 treatment, whereas the budding process remained unaffected and revealed the Rad53 dependence of the extra delay observed after exposure to H_2O_2 of G_1 -phase *apn1Δ apn2Δ* cells. Rad53 phosphorylation observed in G_1 and in G_2 -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 G_1 , S and G_2 phases upon treatment with 0.8 mM H_2O_2 ; (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 G_1 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 H_2O_2 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,

Fig. 7. Hydrogen peroxide treatment of G₁- and G₂-M-synchronized *apn1Δ apn2Δ* cells results in extra Rad53-dependent delays of cell cycle progression. Exponentially growing wild-type (MCM185), *apn1Δ apn2Δ* (MCM283) and *apn1Δ apn2Δ rad53Δ* (MCM332) cells were synchronized in G₁ with α -factor (A and B), or in G₂-M with nocodazole (C and D), released from the blocking factors, and immediately treated with 0.8 mM H₂O₂ or left untreated. (A and C) FACS analysis of cells synchronized in G₁ or G₂-M phases and either treated with 0.8 mM H₂O₂ (+) or left untreated (-). Aliquots were taken from exponentially growing cells (exp), α -factor- or nocodazole-synchronized cells (α F, noc) and at the indicated times after release from the blocking factors. (B and D) Percentage of budded or unbudded cells in α -factor- or nocodazole-synchronized cell cultures either treated with 0.8 mM H₂O₂ at $t = 0$ min (closed symbols) or left untreated (open symbols), at the indicated times after release from the blocking factors.

Rad53 phosphorylation was found to be similar in *apn1Δ apn2Δ rad14Δ* and in *apn1Δ apn2Δ* 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 H₂O₂ delays cell cycle progression in G₁, S and G₂ 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 H₂O₂ treatment specifically during S phase. Finally, we demonstrated that the lack of Rad53 phosphorylation after H₂O₂

treatment in the G₁ and G₂ 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 H₂O₂ 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_2O_2 (Szatrowski 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_2O_2 is dependent upon Rad53. The mechanisms responsible for the delays occurring in the G_1 and G_2 phases after H_2O_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 *sod1Δ* 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_2O_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_2O_2 to S-phase cells could be explained by an effect of low concentrations of H_2O_2 on ribonucleotide reductase (RNR) activity through depletion of reducing agents. However, Rad53 phosphorylation in S phase upon exposure to H_2O_2 is more intense in *apn1Δ apn2Δ* cells than in wild-type cells, which strongly suggests that DNA lesions are induced by H_2O_2 in this phase. Furthermore, the observation that Rad53 phosphorylation in wild-type cells treated with H_2O_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 (Pelliccioli *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_2O_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_2O_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 recom-

bination intermediates generated by the stalling of the replication fork) recognizable by DNA damage sensors (Foiani *et al.*, 1998). Thirdly, mechanisms capable of sensing H_2O_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_2O_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 Siede *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_2O_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_2O_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_2O_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_2O_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-

Nucleotide Excision Repair (NER)

Base Excision Repair (BER)

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.

point proteins or processed further by alternative pathways into intermediates able to activate the DNA checkpoints (Figure 8). Helicases or nucleases might be involved in the alternative processing of Apn1/Apn2 substrates. Interestingly, Xiao and Chow (1998) recently showed not only that mutations in the NER genes *RAD1*, *RAD2*, *RAD4* and *RAD10* and in the BER gene *APN1* are synergistic with respect to killing by methyl methanesulfonate (MMS), but also that among the *rad apn1Δ* mutants affected in both the NER and the BER pathways, the *rad1Δ apn1Δ* and *rad10Δ apn1Δ* double mutants exhibit unique phenotypes with slow growth rates and additional synergistic sensitivity to MMS. 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 co-lethal 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/Apn2 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*, *trp1-Δ63*,

his3-Δ200, *leu2Δ-1*, *bar1Δ::LEU2*), a YPH499 derivative in which the *BAR1* 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 *rad9Δ::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'-TAGAAAAGAGCATAGTGAGAAATCTTCAACATCAGGGCTCGTACGCTGCAGGTCGAC-3') and *RAD9D3* (5'-ATCATTGTCCGTAATATCATCGTGAAGAACAGTGTCCTCGATCGATGAATTCGAGCTCG-3'); *RAD9D5URA* (5'-CGC-CATAGAAAAGAGCATAGTGAGAAAATCTTCAACATCAGGGCTA-CGTGATTGCTTAAGAATT-3') and *RAD9D3URA* (5'-TCT-AACCTCAGAAAATAGTGTTGTATATATCATTGTCCGTAATATCG-TAGTTTCTGGTTTTTAAAT-3'); *RAD17D5* (5'-CAGAACGGT-GTGGAAACAAAGTAGTTGAAGGATTTCAACTCGTACGCTGCAGGTCGAC-3') and *RAD17D3* (5'-TGAATGAAGTTCTCGGTT-TTCTGCGATGCTGGATATTGACATCGATGAATTCGAGCTCG-3'); *RAD53D5* (5'-GAGAGAATAGTGAGAAAAGATAGTGTACACAA-CATCACTAAAAAGCTTCGTACGCTGCAGGTCGAC-3') and *RAD-53D3* (5'-CTCTCTTAAAAAGGGGAGCAGCATTTCTATGGGTATT-TGTCCTTGATCATCGATGAATTCGAGCTCG-3'); *APN1D5* (5'-CAAAACGCAACATTAATAAGCTTTTGGCATATCGGAACCATC-GTAGCTTCGTACGCTGCAGGTCGAC-3') and *APN1D3* (5'-ATA-ATCTACAAAAATGATTACGTATTTAAAAATCTTCTCGTTCA-TCATCGATGAATTCGAGCTCG-3'); *APN2D5* (5'-AAGCTATTT-CACCGTAAAGAAAATCCCTTTCCTTGTCAGGACACTACGTGA-TTTGCTTAAGAATT-3') and *APN2D3* (5'-AGAAAGTGTTTTATT-CTCCCAAAATATCAGCTGACGTTTTCATATGATGTTCTGGT-TTTTAAAT-3'); and *RAD14D5* (5'-AAAAAGAGTTTGGATCTT-CGTAGTGAAGGTATCGAACGTAACGCTACGTGATTTGCTTA-AGAATT-3') and *RAD14D3* (5'-CTTATTATGACTTTCTTGTTA-TATTCTTATATACATAACCAACATGATTTCTGGTTTTTAA-AT-3'), respectively, as primers. One-step replacements of the *RAD9*, *RAD17*, *RAD53*, *APN1* and *RAD14* coding regions with the *KanMX4* cassette or *K.lactis* *URA3* gene were carried out by transforming MCM185 with the *rad9Δ::kanMX4*, *rad17Δ::kanMX4*, *rad53Δ::kanMX4*, *apn1Δ::kanMX4* and *rad14Δ::URA3* PCR products to give rise to strains MCM215, MCM188, MCM196, MCM279 and MCM305, respectively. The *apn1Δ apn2Δ* double mutant MCM283 was constructed by transforming the *apn1Δ* single mutant MCM279 with the *apn2Δ::URA3* PCR product. Ura⁻ mutants were selected from the MCM283 strain by plating on 5-fluoro-orotic acid (5-FOA)-containing plates and transformed further with the *rad14Δ::URA3* PCR product to

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

Strain	Genotype	Reference
YPH499	<i>MATa</i> , <i>ura3-52</i> , <i>lys2-801amber</i> , <i>ade2-101ochre</i> , <i>trp1-Δ63</i> , <i>his3-Δ200</i> , <i>leu2-Δ1</i>	Sikorski and Hieter (1989)
MCM185	as YPH499 <i>bar1Δ::LEU2</i>	this study
MCM188	as MCM185 <i>rad17Δ::kanMX4</i>	this study
MCM189	as MCM185 <i>mec1Δ::HIS3</i> + pBAD70 (2μ, <i>TRP1</i> , <i>GAP-RNR1</i>)	this study
MCM196	as MCM185 <i>rad53Δ::kanMX4</i> + pBAD70 (2μ, <i>TRP1</i> , <i>GAP-RNR1</i>)	this study
MCM215	as MCM185 <i>rad9Δ::kanMX4</i>	this study
MCM247	as MCM185 <i>rad17Δ::kanMX4 rad9Δ::URA3</i>	this study
MCM258	as MCM185 <i>rad53Δ::kanMX4</i> + pBAD70 (2μ, <i>TRP1</i> , <i>GAP-RNR1</i>) + pJA92/ <i>rad53-21</i> (<i>CEN</i> , <i>URA3</i> , <i>rad53-21</i>)	this study
MCM279	as MCM185 <i>apn1Δ::kanMX4</i>	this study
MCM283	as MCM185 <i>apn1Δ::kanMX4 apn2Δ::URA3</i>	this study
MCM305	as MCM185 <i>rad14Δ::URA3</i>	this study
MCM309	as MCM185 <i>apn1Δ::kanMX4 apn2Δ::ura3 rad14Δ::URA3</i>	this study
MCM332	as MCM185 <i>apn1Δ::kanMX4 apn2Δ::URA3 rad53Δ::kanMX4</i> + pBAD70 (2μ, <i>TRP1</i> , <i>GAP-RNR1</i>)	this study

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 *mec1Δ::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 *rad53-21* (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 *AflIII* 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 *rad53-21* 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* mutant MCM332 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 were grown at 30°C in YPD medium (1% yeast extract, 2% bactopectone, 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 Stratilinker 1800 (Stratagene).

Analysis of Rad53 phosphorylation

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

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