Hydrogen Peroxide Causes RAD9-dependent Cell Cycle Arrest in G₂ in Saccharomyces cerevisiae whereas Menadione Causes G₁ Arrest Independent of RAD9 Function*

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This study shows differences at the level of cell cycle arrest between the response of yeast cells to hydrogen peroxide and superoxide stress. These include both cell cycle phases at which arrest occurs and the involvement of the RAD9 checkpoint gene. Wild-type and rad9 cells were treated with hydrogen peroxide or the superoxide-generating agent menadione. rad9 mutants were up to 100-fold more sensitive to hydrogen peroxide but not affected in their resistance to menadione. Hydrogen peroxide caused G₂-phase arrest, whereas menadione-treated cells arrested in G₁. G₂ arrest, induced by methyl 2-benzimidazil carbamate, increased cellular resistance to hydrogen peroxide but not to menadione. G₁ arrest mediated by α-factor caused an increase in survival of wild-type cells treated with menadione but not with hydrogen peroxide. A cdc28 mutant arrested in G₁ was significantly more sensitive to hydrogen peroxide than other cdc mutants arrested in later phases, including G₂. rad9 cells have normal stationary phase resistance to hydrogen peroxide, the ability to adapt to it, glutathione content and induction of genes via the stress responsive element. Although rad9-dependent G₂ arrest is important, other rad9-dependent factors may be involved in the resistance of cells to hydrogen peroxide since arrest in G₂ did not make rad9 cells fully resistant.

Oxidative stress generated by hydrogen peroxide or the superoxide anion causes an adaptive response in the yeast, Saccharomyces cerevisiae. Cells pretreated with a low dose of these reactive oxygen species (ROS) subsequently adapt to become more resistant to higher doses (1–3). Study of the mechanisms that lie behind this response has focused mainly on the role of antioxidant enzymes and detoxifying molecules such as glutathione.

Several genes whose products either directly eliminate ROS or form part of the glutathione synthesis pathway are transcriptionally regulated by oxidative stress, e.g. CTT1 encoding cystosolic catalase, SOD1 and SOD2 encoding Cu, Zn- and Mn-superoxide dismutase respectively. GSH1 encoding γ-glutamylcysteine synthetase, GLR1 encoding glutathione reductase, TRX2 encoding thioredoxin (a scavenger of ROS), and SSA1 encoding a stress-inducible heat shock protein (4–9).

Transcription factors and their target DNA sequences have been identified in genes that respond to oxidative stress: Msn2p and Msn4p function via the stress responsive element (STRE) (10), and the Yap1p binding site resembles the SV40 AP-1 binding sequence (8, 11). These factors clearly play an important role in the stress response of yeast cells since S. cerevisiae mutants deleted for MSN2/4 or YAP1 are hypersensitive to various types of stress including treatment with hydrogen peroxide.

Despite the fact that oxidative stress can cause damage to many cellular components including proteins, lipids, and DNA, few studies have considered the potential importance of cell cycle arrest in response to DNA damage caused by hydrogen peroxide or superoxide. Both prokaryotic and eukaryotic cells respond to DNA damage (caused by UV, x-, or γ-irradiation) by delaying cell cycle progression to allow repair of damage as well as preventing segregation of affected chromosomes. In Escherichia coli, the SOS system controls cell cycle arrest and coordinates the activation of approximately 20 genes including those involved in nucleotide excision repair, recombination and mutagenesis (12, 13). An analogous system is thought to exist in S. cerevisiae, although the precise mechanism is not well understood.

Checkpoints can be defined as negative controls that cause cell cycle arrest in response to cellular damage. RAD9 is the best characterized of the S. cerevisiae checkpoint genes and has been shown to act at G₁, S and G₂ phases (14–16). The molecular function of RAD9 is unknown but cells that are defective in this gene exhibit increased sensitivity to DNA damaging agents. Treatment with the microtubule inhibitor methyl 2-benzimidazil carbamate (MBC), which blocks cells in the G₂ phase, reversed this sensitivity indicating that RAD9 functions in cell cycle arrest and is not a DNA repair enzyme (16, 17). Curiously, however, MBC treatment did not rescue rad9 cells exposed to UV radiation, and this pointed toward a further function for RAD9 other than cell cycle arrest. Aboussekhra et al. (17) have recently shown that RAD9 is involved in transcriptional induction of genes responsible for multiple DNA metabolism/repair pathways, and postulated that the response pathways controlled by RAD9 may be the functional equivalents of the E. coli SOS response.

Neither hydrogen peroxide nor the superoxide radical can directly attack DNA (18), but DNA strand breaks arise when cells are exposed to these reagents due to the production of the highly reactive hydroxyl radical (19, 20). The relative importance of the repair of this damage in the general response of yeast cells to oxidative stress remains unclear. However, slow proliferation of a S. cerevisiae mutant strain lacking Cu/Zn superoxide dismutase (sod1) was due to a 2-fold increase in time spent in G₁ phase (21). Under 100% O₂ the mutant permanently arrested in G₁ due to inhibition of transcription of the
autoregulated G1 cyclins, CLN1 and CLN2. Moreover, treatment with paraquat, a superoxide-generating compound, resulted in pronounced G1 arrest in G1-synchronized yeast cells (22). Interestingly, cell cycle arrest under these circumstances was found to be independent of the RAD9 gene.

This study investigated the role of RAD9 cell cycle arrest in the response to hydrogen peroxide and the superoxide-generating agent, menadione. These compounds have been shown to elicit overlapping but different responses in S. cerevisiae (2, 3, 23). We have shown that a rad9 mutation rendered cells 10–100-fold more sensitive to hydrogen peroxide but, surprisingly, did not affect resistance to menadione. Since RAD9 is involved in G1, S, and G2 arrest in response to DNA damage, arrest induced by MBC and the mating pheromone, α-factor, as well as cell cycle arrest in temperature-sensitivecdc mutants was used to investigate which checkpoint might be involved. Overall, capacity of the rad9 mutant to resist oxidative stress was also studied by assessing resistance in stationary phase, glutathione levels, ability to mount an adaptive response, and transcriptional activation through STRE elements. Our results show that the RAD9 function defines a fundamental difference between the response to peroxide and superoxide stress. In addition, the acute sensitivity of the rad9 mutant to hydrogen peroxide, which is not fully rescued by MBC, indicates that a further rad9-dependent function as well as G2 checkpoint arrest may be important for survival during hydrogen peroxide stress.

MATERIALS AND METHODS

Strains and Media—Wild-type yeast strain 7830-2-4a MAta his3 leu2 trpl ura3 and its isogenic mutant 7833-1a MAta his3 leu2 trpl ura3 rad9 TRPI were a gift from L. Hartwell; Y382 MATa ura3 leu2 ade2::lacZ were obtained from A. Bender, and strains carrying mutations in CDC4, CDC13, CDC16 and CDC28 each backcrossed several times to wild-type MAta ade2-1 trpl-1 can1-100 leu2-3 leu2-112 his3-11 his3-15 ura3 GAL psi+ or its MAta counterpart were obtained from the Research Institute of Molecular Pathology, Vienna.

Cells were cultured in rich glucose medium (YEPD: 2% D-glucose, 0.17% yeast nitrogen base without amino acids and ammonium sulfate (Difco), 0.5% ammonium sulfate and 50 mg/liter appropriate amino acids). Cells were routinely grown at 30 °C except temperature-sensitive mutants were cultured at 23 °C before being shifted to the restrictive temperature of 37 °C.

Hydrogen Peroxide/Menadione Treatments—Cells were grown overnight to an A600 of 0.5 at 30 °C with shaking in the appropriate medium. Temperature-sensitive mutants were grown at 23 °C overnight and shifted to 37 °C for 4 h before treatment. Hydrogen peroxide or menadione was added directly to the medium when cells were cultured in either YEPD or SD medium. When treatments were carried out in buffer, cells were harvested by centrifugation at 25 °C (4 × 10^7 × g for 5 min), washed, and resuspended in an equal volume of 100 mM potassium phosphate, pH 7.4. Samples (5 ml) were treated with hydrogen peroxide or menadione as indicated. In all cases, appropriate dilutions were made before plating cells on YEPD agar plates to obtain viable counts.

YEPD Agar Plate Test of Resistance—Cells were grown to stationary phase and diluted to an A600 of 3 and 0.3. Drops (4 μl) of each culture were placed on YEPD agar containing appropriate concentrations of hydrogen peroxide, tert-butyl hydroperoxide or menadione. Plates were dried, incubated at 30 °C for 2 days, and photographed.

Measurement of DNA Content—Cells were fixed in 70% cold ethanol and stored overnight at 4 °C. Samples (10 ml) were washed and resuspended in 1 ml of 50 mM sodium citrate buffer, pH 7.5. RNAase (25 μl of 20 mg/ml solution) was added, and the mixture was incubated for 1 h at 50 °C to incubate for 1 h at 50 °C. Propidium iodide (50 μg) in 50 mM sodium citrate buffer (1 ml) was added and samples were incubated in the dark at 4 °C overnight. Flow cytometry analysis of at least 100,000 cells was carried out for each sample in a MoFlo analyzer (Cytomation).

Cell Cycle Arrest and Treatment—Cells were grown overnight in the appropriate medium to an A600 of 0.5 at 30 °C with shaking, divided, and exposed to either α-factor or MBC. α-factor was purchased from Sigma and added at a final concentration of 0.5 μg/ml. Stock MBC, purchased from Aldrich, was prepared in a 10 mg/ml solution in MeSO and added to cells at a final concentration of 100 μg/ml. An equal volume of MeSO (without MBC) was added to the MBC treatment control culture. All cultures were incubated at 30 °C for one generation after which treatments were carried out as indicated above in the presence of either α-factor or MBC, and cell survival was monitored as described previously.

Adaptation to Hydrogen Peroxide—Overnight cultures grown in YEPD to an A600 of 0.5 at 30 °C were exposed to hydrogen peroxide pretreatment doses at the concentrations indicated. Incubation at 30 °C (22). Interestingly, cell cycle arrest under these circumstances was found to be independent of the RAD9 gene.

RESULTS

Effect of a Cell Cycle Checkpoint Mutation on Hydrogen Peroxide and Menadione Resistance—Cells that are sensitive to DNA damage may also be more sensitive to peroxide or menadione stress even if other factors involved in the oxidative stress response are intact. Cells defective in the RAD9 checkpoint gene are sensitive to radiation due to their failure to induce cell cycle arrest in response to DNA damage. To determine whether this arrest malfunction would render cells more sensitive to oxidative stress, a wild-type strain and its isogenic rad9 derivative were grown to an A600 of 1 in YEPD medium and treated with various concentrations of hydrogen peroxide over a 2-h period. rad9 cells were 10–100 times more sensitive than the wild-type at concentrations of hydrogen peroxide above 1 mM (Fig. 1). At concentrations of 2 mM or more, rad9 cell survival was monitored as described previously. The difference in sensitivity of wild-type and rad9 cells to peroxide was also observed when cells were exposed to either hydrogen peroxide or tert-butyl hydroperoxide on solid YEPD medium (Fig. 2). Interestingly, no difference in the sensitivity of the strains to menadione was detected with both being viable.
a time medium but unable to grow at 2 mM. However, when cells were treated in liquid YEPD medium they were insensitive to much higher concentrations of menadione and other means of making a quantitative assessment of survival were sought.

Menadione treatment in potassium P, buffer substantially reduces cell viability at relatively low concentrations (3). Therefore, the wild-type strain and its isogenic rad9 derivative were treated with various concentrations of either hydrogen peroxide or menadione for 1 h in 100 mM P, buffer (Fig. 3). Compared with cell survival rates in rich medium (cf. Fig. 1), both strains were substantially more sensitive to these reagents when exposed in buffer. Nevertheless, a 10–100-fold difference was again observed between wild-type and rad9 cells treated with hydrogen peroxide (Fig. 3A). In contrast, however, rad9 cells were no more sensitive to menadione than wild-type cells (Fig. 3B).

Cell Cycle Arrest in Response to Reactive Oxygen Species—Since rad9 cells fail to arrest either in G1, S, or G2 phase, it was important to assess at which stage of arrest cells acquired resistance to hydrogen peroxide. In addition, since paraquat (also a superoxide generator) induces G1 arrest (22), it was of interest to know whether menadione could do so.

Survival of menadione- or peroxide-treated cells in defined SD medium was intermediate between survival in YEPD and buffer (results not shown). This medium was therefore used for both hydrogen peroxide and menadione treatments to allow normal cell cycle progression upon exposure to low doses of the reagents and enable comparison between the treatments. Cells were grown to A600 of 1 and the culture split into three. Over a 3-h period, one aliquot was allowed to grow without treatment (control) and the second and third aliquots were treated with hydrogen peroxide or menadione, respectively. The proportion of cells in G1 or G2 phase was inferred from flow cytometry analysis in which DNA content of cells was measured (Fig. 4). Menadione, like paraquat, caused arrest in G1, and since rad9 cells are not particularly sensitive to menadione, this arrest is relatively independent of RAD9 function. Hydrogen peroxide treatment, however, caused cells to arrest in G2, which appears therefore to be a RAD9-dependent effect.

Strain Y382 was used for the flow cytometry analysis discussed above but since the wild-type and isogenic rad9 strains have a “clumpy” phenotype, flow cytometry results were very variable when methods to separate clumps were employed (e.g., sonication). Therefore microscopic examination of cell morphologies was carried out to confirm the previous experiment and extend it to the rad9 mutant. Cultures of the wild-type and rad9 isogenic strains grown in minimal medium were treated with a range of concentrations of menadione (from 0.5 to 10 mM) and hydrogen peroxide (0.01–2 mM), and viability was estimated. For those cultures in which the treatment led to

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**FIG. 1.** Sensitivity of wild-type and rad9 cells to hydrogen peroxide treatment in YEPD medium. Exponential phase wild-type and rad9 cells growing in YEPD medium were treated with hydrogen peroxide (mM H2O2: □, 0; ○, 0.2; △, 0.4; △, 1; ○, 2; ●, 4) over a 2-h period. Samples were diluted and plated on YEPD solid medium to monitor cell viability. Less than 0.001% survival of the rad9 strain was recorded after 4 mM hydrogen peroxide treatment over 2 h. Data are the mean of duplicates from a representative experiment and are calculated as a percentage of the number of cells present at time 0.

**FIG. 2.** Sensitivity of wild-type and rad9 cells to ROS. Drops (4 μl) of wild-type and rad9 cultures at A600 (OD600) of 0.3 and 3 were placed on YEPD agar plates containing either hydrogen peroxide, tert-butyl hydroperoxide, or menadione at the concentrations indicated. Plates were incubated for 2 days at 30 °C and immediately photographed.

at 1 mM concentration but unable to grow at 2 mM. However, when cells were treated in liquid YEPD medium they were insensitive to much higher concentrations of menadione and other means of making a quantitative assessment of survival were sought.

Menadione treatment in potassium P, buffer substantially

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**FIG. 3.** Sensitivity of wild-type and rad9 cells to hydrogen peroxide and menadione in P, buffer. YEPD-grown cells were re-suspended in P, buffer and treated with various concentrations of hydrogen peroxide and menadione for 1 h. Cell viability was monitored by plating appropriate cell dilutions on YEPD solid medium. □ and ○ represent wild-type and rad9 survival, respectively, calculated as a percentage of the number of cells present prior to treatment. Average values for triplicates are represented as the standard deviations indicated.
about 90% survival (measured as cells capable of further growth), at least 400 cells were counted, and flow cytometry results were confirmed (Table I). Cells in G₁ were identified by their large unbudded shape, whereas cells with buds more than two-thirds the size of the mother cell were considered to be in G₂ (17). The ratio of cells in G₁:G₂ for the wild-type decreased 3-fold when they were treated with hydrogen peroxide (0.25 mM) and increased 2-fold when treated with menadione (4 mM) for 2 h. The rad9 cells also arrested in G₁ in response to menadione treatment with the G₁:G₂ ratio increasing about 2-fold, however, only a small difference in the number of cells arrested in G₂ phase was noted in rad9 cells treated with 0.05 mM hydrogen peroxide.

Effect of G₁ and G₂ Arrest on Resistance to Hydrogen Peroxide and Menadione—Since hydrogen peroxide and menadione treatment cause cells to arrest in G₁ and G₂, respectively (26, 27), the experiment was conducted such that comparison could be made between treatments, which resulted in similar survival levels (2 mM hydrogen peroxide for the wild-type and 1 mM hydrogen peroxide for rad9 cells) and similar treatment concentrations (i.e., 2 mM treatments for both cell types). Survival was calculated as a percentage of cells present before treatment with the ROS.

When wild-type cells were arrested in G₁ by α-factor, they became more sensitive to hydrogen peroxide and more resistant to menadione (Fig. 5, A and B; note the difference in axes needed to show this effect). However, the opposite effect was noted when cells were arrested in G₂ by MBC treatment. In an asynchronous culture, cells are distributed in the different stages G₁, G₂, and M, and so a number of cells will be at the appropriate stage to render them more resistant to the particular oxidant. Therefore, for hydrogen peroxide treatment, arresting cells in G₁ led to increased sensitivity compared with the asynchronous culture in which a higher proportion of cells were in G₂. On the basis of these comparisons, arresting cells in G₁ led to resistance to menadione but not to H₂O₂, whereas arrest in G₂ led to increased resistance to H₂O₂ and not to menadione.

In rad9 cells, a 10–100-fold increase in resistance was noted for hydrogen peroxide-treated cells arrested by MBC in G₂. These results are consistent with the involvement of RAD9 in this resistance. At equivalent doses of peroxide (0.2 mM), arrest of rad9 cells in G₂ did not completely restore resistance of the cells to wild-type levels. This may indicate that there is an additional effect of the rad9 mutation on cell survival; this is discussed later.

Hydrogen Peroxide Resistance of Temperature-sensitive Cell Division Cycle (cdc) Mutants—Many temperature-sensitive mutants have been isolated that arrest at defined points in the cell cycle when shifted to the restrictive temperature. A range of mutants was examined to determine the effect of each mutation on resistance to hydrogen peroxide. Strains tested included cdc28 mutants, which cause arrest at START, the control point at which cells leave G₁ and enter the mitotic cycle (28, 29), cdc4, which arrests after START but prior to separation of the spindle pole bodies (28, 30), and cdc13 and cdc16, which both cause arrest in G₂/M phase (31).

Wild-type and mutant cells were grown overnight at 23 °C and shifted to the restrictive temperature 4 h prior to treatment in the YEPD growth medium. All strains were significantly less sensitive to hydrogen peroxide at the restrictive temperature than at 23 °C (results not shown), which may have been due to the heat shock experienced. Arrest in G₂ (cdc28 mutant) caused cells to be particularly sensitive to hydrogen peroxide, the least sensitive were the cdc13 and cdc16 mutants blocked in G₂/M (Fig. 6), which would be expected if G₂ arrest was needed to repair peroxide damage. Curiously, none of the arrested cdc mutants became more resistant than the wild-type.

**TABLE I**

| Treatment | Strain | G₁:G₂ ± S.D. |
|-----------|--------|--------------|
| Control   | Wild-type | 1:1.13 ± 0.20 |
| Hydrogen peroxide | rad9  | 1:1.24 ± 0.21 |
| 0.25 mM | Wild-type | 1:1.31 ± 0.30 |
| 0.05 mM | rad9  | 1:1.49 ± 0.32 |
| Menadione | Wild-type | 1:0.47 ± 0.05 |
| 4 mM | rad9  | 1:0.53 ± 0.03 |
| 4 mM | rad9  | 1:0.53 ± 0.03 |

FIG. 4. Flow cytometry analysis of cells treated with hydrogen peroxide or menadione. Cells were grown in defined SD medium to A₆₀₀ of 1 and divided into three. One sample was allowed to continue growing without treatment (control) and the others were treated with either hydrogen peroxide (1 mM) or menadione (4 mM). Samples were taken every hour over a 3-h period, and at least 100,000 cells from each sample were analyzed in a Cytometry MoFlo flow cytometer. Peaks represent cells that contain either one or two copies of the genome and are in G₁ or G₂ phase of the cell cycle, respectively.
Hydrogen Peroxide Resistance of Stationary Phase rad9 Cells—
Mutational analysis has shown that stationary-phase cells are in a unique state and are not arrested at a point in the mitotic cell cycle (32). Stationary phase cells are more resistant to several types of stress including oxidative stress (2, 33). If the rad9 sensitivity to hydrogen peroxide is due to its dysfunctional checkpoint arrest then in stationary phase when cells are not undergoing cell division, their resistance to hydrogen peroxide should increase.

The rad9 and wild-type cells were grown to stationary phase in YEPD (a 3-day growth) and treated for 1 h with various concentrations of hydrogen peroxide (Fig. 7). Comparison with data in Fig. 1 (exponential phase cells) reveals that, as shown by others, survival of the wild-type was substantially higher in cells grown to stationary phase. Of more interest, however, is that rad9 cells not only increased their survival rate but became as resistant to hydrogen peroxide as wild-type cells (4 mM hydrogen peroxide treatment killed more than 99.9% of exponential phase rad9 cells but less than 50% of stationary phase cells). The RAD9 function, therefore, does not play a role in the increased resistance of stationary phase cells to oxidative stress.

Assessment of Antioxidant Defense Systems in rad9 Cells—
G2/M arrest by MBC significantly increased survival of rad9 cells but did not restore wild-type survival levels. RAD9 has recently been shown to control not only the cell cycle checkpoint delay but also DNA damage-dependent transcriptional induction of a “regulon” of genes whose products are involved in several DNA repair pathways. This secondary control appears to be independent of the cell cycle (17). It was important therefore to determine whether RAD9 also played a role in induction of the overall response to oxidative stress and whether the increased sensitivity of rad9 cells to hydrogen peroxide treatment reflected an inability to activate the correct stress re-
response in addition to the inability to arrest at the RAD9 checkpoint. Therefore, rad9 cells were assessed for their level of the important antioxidant glutathione; their ability to show an adaptive response to hydrogen peroxide; and induction of transcription through the STRE element.

Glutathione, an ubiquitous thiol which acts as a scavenger of free radicals, is essential for resistance and adaptation to hydrogen peroxide (34, 35). It is the most abundant antioxidant molecule (36) in the cell and was thus a good candidate for investigation of the intrinsic ability of rad9 cells to eliminate free radicals prior to them reaching the nucleus. Moreover, the ratio of reduced to oxidized glutathione is an indication of the capacity of cells to deal with oxidative stress. Glutathione content of exponential phase wild-type and rad9 cells was analyzed in YEPD-grown cultures. Both oxidized and reduced glutathione levels in wild-type (12 ± 2 and 559 ± 95 nmol/10^9 cells, respectively) and rad9 (13 ± 3 and 473 ± 77 nmol/10^9 cells, respectively) were similar and glutathione content, therefore, appears to be unrelated to RAD9.

The adaptive response to oxidants includes transcriptional induction of genes encoding protective proteins. There are some difficulties interpreting the results when assessing whether rad9 cells were impaired in this protective mechanism due to the large differences in sensitivity to hydrogen peroxide compared with wild-type cells. It was estimated from kill curve data that a 0.1 mM pretreatment of wild-type cells may be equivalent to a 0.05 mM pretreatment of rad9 cells. Pretreatments were carried out for 1 h in YEPD, and concentrations varied from 0.05 to 0.2 mM hydrogen peroxide while the challenge treatment (1 mM for 1 h) in 100 mM P_i buffer) was kept constant (Fig. 8). Despite different sensitivities of the cell types, the magnitude of the adaptive response was comparable when pretreatments were equivalent i.e. a 3.5-fold increase in survival for both strains upon pretreatment with 0.1 mM peroxide; 6.4- and 9.5-fold survival increase for wild-type and rad9 strains, respectively, when pretreated with 0.2 mM peroxide. It was concluded therefore that there was no impairment of the adaptive response per se in rad9 cells despite their increased sensitivity to hydrogen peroxide.

Transcription of several stress-related genes including catalase is induced through the STRE element (10), and since RAD9 can act as a transcriptional regulator (17) it was important to test whether the response was mediated through this element. A plasmid containing seven STRE elements in different orientations 5’ to minimal LEU2 promoter::lacZ reporter construct was inserted in single copy into wild-type and rad9 cells. Transformants were treated for 90 min with either 0.4 or 1 mM hydrogen peroxide in YEPD and β-galactosidase activity measured after 45 and 90 min of exposure (results not shown). At both times in both cell types, 0.4 mM treatment induced higher expression of β-galactosidase than 1 mM treatment. No difference, however, was observed between levels of induction of β-galactosidase in wild-type and rad9 cells, and it was concluded, therefore, that the transcriptional response of genes to oxidative stress mediated by the STRE element was intact in rad9 cells.

DISCUSSION

In addition to their UV, x-, and γ-radiation sensitivity, this study has demonstrated that cells defective in RAD9 are also extremely sensitive to hydrogen peroxide compared with wild-type cells. RAD9 checkpoint arrest in response to DNA damage operates in G_1, S, and G_2 phases, although the mechanism by which it does so is not entirely clear (14–16). Flow cytometry analyses revealed that hydrogen peroxide arrests cells in G_2 phase, and it is suggested, therefore, that this is at least partly due to the cell cycle arrest function of RAD9.

Hydrogen peroxide does not directly damage DNA but forms the hydroxyl radical that can then attack bases, sugars, and the phosphate backbone of the DNA helix (13). It has been estimated that approximately 65% of DNA damage caused by ionizing radiation is also due to hydroxyl radical attack, whereas approximately 35% is due to direct ionization effects (37). Hence, it could be anticipated that the responses to hydrogen peroxide and ionizing radiation would overlap considerably.

MBC treatment can functionally substitute for G_2 arrest and rescue rad9 cells treated with x- and γ-radiation (16, 17). However, MBC treatment prior to hydrogen peroxide challenge only partially rescues rad9 cells. This may imply that whereas ionizing radiation-sensitivity of rad9 cells is entirely due to the cell cycle arrest function of RAD9, the RAD9-dependent response to hydrogen peroxide relies on another function of RAD9 other than G_2 arrest. Although RAD9 transcript is not induced by UV or x-irradiation (38, 39) and transcriptional induction is

![Graph 7](image7.png)

**Fig. 7.** Survival of stationary phase rad9 cells compared with wild-type. Wild-type and rad9 cells were grown over 3 days in YEPD to stationary phase and treated with hydrogen peroxide at the concentrations indicated over a 2-h period. Round symbols (○, ●) represent stationary phase cell survival; square symbols ( □, ■) represent exponential phase; ○ and ● represent wild-type; and □ and ■ represent rad9 cell survival. Cell viability was measured by plating of appropriate dilutions on YEPD medium. Average values for triplicates are represented as the standard deviations shown. At concentrations ≥ 4 mM hydrogen peroxide, rad9 cell survival in exponential phase was less than 0.001% after 2 h treatment.

![Graph 8](image8.png)

**Fig. 8.** Adaptation of rad9 cells to hydrogen peroxide treatment. YEPD-grown wild-type and rad9 cells were pretreated with hydrogen peroxide at the concentrations indicated for 1 h in YEPD. Cells were subsequently challenged with a 1 mM treatment in P_i buffer for 1 h. Cell viability was measured by plating appropriate cell dilutions on YEPD solid medium. Data are the mean of triplicates with standard deviations indicated.
Expressions of genes involved in DNA repair pathways, including those encoding DNA repair enzymes, are upregulated in response to DNA damage. This indicates that the cell cycle arrest induced by DNA damage is not only a mechanism for cell survival but also a means to repair and maintain the integrity of the genome. The cell cycle arrest function is regulated at transcriptional and post-transcriptional levels, with evidence suggesting that specific transcription factors and miRNAs play roles in this process. For instance, the transcription factor E2F is activated upon UV irradiation, leading to cell cycle arrest in G1 phase. This transcriptional response is coordinated with the expression of miRNAs, such as miR-210, which targets the E2F transcription factor.

In summary, the cell cycle arrest response to DNA damage is a highly coordinated process involving multiple layers of regulation. This response is essential for maintaining genomic stability and ensuring the survival of the organism. Future research should focus on understanding the mechanisms underlying these regulatory pathways and how they are disrupted in cancer and other diseases.

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