Robust G1 checkpoint arrest in budding yeast: dependence on DNA damage signaling and repair

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Summary

Although most eukaryotes can arrest in G1 after ionizing radiation, the existence or significance of a G1 checkpoint in *S. cerevisiae* has been challenged. Previous studies of G1 response to chemical mutagens, X-ray or UV irradiation indicate that the delay before replication is transient and may reflect a strong intra-S-phase checkpoint. We examined the yeast response to double-stranded breaks in G1 using γ irradiation. G1 irradiation induces repair foci on chromosome spreads and a Rad53 band shift characteristic of activation, which suggest an active DNA damage response. Consistent with a G1 arrest, bud emergence, spindle pole duplication and DNA replication are each delayed in a dose-dependent manner. Sensitivity to mating pheromone is prolonged to over 18 hours when G1 cells are lethally γ or UV irradiated. Strikingly, G1 delay is the predominant response to continuous γ irradiation at a dose that confers no loss of viability but delays cell division. Like the G2/M checkpoint, G1 delay is completely dependent on both *RAD9* and *RAD24* epistasis groups but independent of *POLε*. Lethally irradiated *rad9* mutants rapidly exit G1 but perform a slow S phase, whereas *rad17* and *rad24* mutants are completely arrest deficient. Distinct from γ irradiation, G1 arrest after UV is *RAD14* dependent, suggesting that DNA damage processing is required for checkpoint activation. Therefore, as in the yeast G2/M checkpoint response, free DNA ends and/or single-stranded DNA are necessary and sufficient to induce a bona fide G1 checkpoint arrest.

Key words: DNA damage checkpoint, DNA repair, G1 phase, *Sacchromyces cerevisiae*, Genetics, Metabolism, Gamma radiation, Cell cycle, *RAD9*

Introduction

Eukaryotic cells regulate the decision to commit to each new cell cycle in mid G1 (Donjerkovic and Scott, 2000; Planas-Silva and Weinberg, 1997). This transition, called the restriction point or Start (Levine et al., 1995; Toone et al., 1997), is the major target of growth factors and nutritional signaling and is promoted through the activation of the G1-phase cyclin-dependent kinase (CDK). Given the importance of this transition, it is not surprising that many cells preferentially arrest in G1 after DNA damage (Bartkova et al., 1997) by inhibiting CDK activity. This G1 arrest allows repair before mutations can be duplicated in S phase or fragmented chromosomes are separated during mitosis.

Recent data suggest that DNA damage is processed by endonucleases to reveal single strands (Garvik et al., 1995; Lee et al., 1998; Lydall and Weinert, 1995). These strands are then detected by proteins implicated both in repair and signaling, such as the tumor suppressors BRCA1 and Nibrin (Scully et al., 1997; Zhong et al., 1999). Complexes of these and other factors that form upon damage induction are thought to mark the sites of damage. The phosphoinositol-3 kinase homologues ATM and ATR, once recruited to these foci, phosphorylate and upregulate downstream effectors, including the dual-specificity kinase CHK2 (Cortez et al., 1999; Matsuoka et al., 2000; Tibbetts et al., 2000). Subsequent phosphorylation of the unstable transcription factor p53 promotes its accumulation (Chehab et al., 2000; Hirao et al., 2000), leading to expression of the $p21^{CIP1}$ CDK inhibitor, preventing onset of replication (Li et al., 1994; Waldman et al., 1995). Cells lacking ATM, p53 or p21 progress into S phase unabated by DNA damage.

The correspondence between bud morphology and cell cycle progression in *S. cerevisiae* (Lew et al., 1997) has made yeast a powerful system for genetic analysis of the cell cycle (Nasmyth, 1996) and DNA damage checkpoints (Weinert, 1998b). Passage through Start occurs before bud emergence, when cells become irreversibly committed to cell division. Exit from G1 is also shown by activation of the yeast Clb5,6-Cdc28 CDK complex, spindle pole body duplication, onset of DNA synthesis and loss of sensitivity to the yeast mating pheromone, alpha factor (αf). Subsequent bud growth continues through S phase and mitosis. Return to G1 is marked by cytokinesis and bud separation. Classic studies of DNA damage checkpoint arrest in *S. cerevisiae* revealed that irradiated yeast cells accumulate with a large-budded morphology (Weinert and Hartwell, 1988). This S phase or G2/M arrest depends on DNA damage sensors that activate the ATM homologue *MEC1* and the CHK2 homologue *RAD53* (Longhese et al., 1998; Weinert, 1998a). Like other eukaryotes, yeast process DNA damage to reveal single strands (Garvik et al., 1995) that are then bound by the RPA protein Rfa1p to initiate the DNA damage signal (Pellicioli et al., 2001). The RFC homologue *RAD24* and a PCNA-like complex composed of *MEC3*, *RAD17* and *DDC1* (Kondo et al., 1999) may recognize these lesions (Durocher and Jackson, 2001). This *RAD17* epistasis group is required for all known DNA damage checkpoints in budding yeast. DNA damage in S phase activates a checkpoint dependent upon the

Polε *POL2* that mediates delays in fork progression and origin firing. *RAD9*, though required for the G2/M checkpoint, has not been shown to function in the DNA replication checkpoint. The *RAD17* pathway, in concert with *RAD9* or *POLε* (de la Torre-Ruiz et al., 1998; Navas et al., 1996), may recruit Mec1p, promoting its phosphorylation of Rad53p (Sanchez et al., 1996).

En route to the large-budded arrest, yeast perform a *RAD17*- and *RAD9*-dependent delay in bud emergence and DNA replication after X-ray-, UV- and MMS-induced DNA damage (de la Torre-Ruiz et al., 1998; Siede et al., 1994; Siede et al., 1993). Stoichiometric inhibitors of Cdc28 exist in yeast; however there are no sequence homologues to p53 or p21. Instead, *MEC1*-dependent activation of Rad53p in G1 (Pellicioli et al., 1999) delays expression of G1 cyclins to slow activation of Cdc28p (Sidorova and Breeden, 1997). Nonetheless, the existence of a bona fide G1 arrest in response to DNA damage has recently come into question (Neecke et al., 1999). Neecke and colleagues observed that UV irradiation in G1 induced a prolonged arrest in mutants lacking the excision repair gene *RAD14*. However, although these *rad14Δ* cells maintain a 1N DNA content, mimicking a G1 arrest, they have entered S phase in the presence of DNA damage as demonstrated by activation of early origins. This arrest is *RAD9* independent, consistent with an S-phase delay. Progression into the cell cycle in the face of unrepaired DNA damage throws doubt on the significance or existence of even the previously described transient G1 checkpoint delay in budding yeast.

Using ionizing radiation, we confirmed that DNA damage in G1 delays both bud emergence and spindle pole body duplication and extends the window of sensitivity to αf. This G1 arrest is dose dependent and requires *RAD9* but not *POLε*. This arrest is not transient. Induced by lethal γ or UV irradiation, the *RAD9*- and *RAD17*-dependent G1 checkpoint arrest can be maintained over 18 hours. In turn, at tolerable levels of γ irradiation, haploid yeast preferentially arrest in G1 without loss of viability. This G1 arrest after γ irradiation is prolonged by defects in DSB (double-strand break) repair. Surprisingly, *rad14* mutants arrest in G1 after γ irradiation but demonstrate a G1 checkpoint defect after UV. Since UV does not directly produce breaks, failure to excise crosslinks may effectively mask DNA damage until replication through the lesion creates a single strand and initiates a DNA damage signal. This model reconciles the apparent conflicts in previous studies of *S. cerevisiae* G1 DNA damage checkpoint response.

Materials and Methods

Strains, microbiological and cytological techniques

Strains were constructed from W303-1A (*MAT**a** ade2-1 can1-100 ura3-1 leu2-3,112 his3-11,15 trp1-1*). Gene knockouts, introduction of 13Myc tags and the C-terminal truncation for *polε-12::kan*^{R} (Navas et al., 1995) were performed by PCR-based gene modification (Longtine et al., 1998). *polε-12::kan*^{R} mutants identified by PCR were confirmed by hydroxyurea sensitivity. *RAD17*, *RAD24* and *RAD9* were disrupted with pDL183, pDL225 (Lydall and Weinert, 1997) and pTWO39 (Siede et al., 1993), respectively. Yeast media and flow cytometry were performed as previously described (Ahn et al., 1999). Nocodazole (USB) was used at 5 μg/ml from a 1000× stock in DMSO. Yeast mating peptide (αf, Research Genetics) was used at 5 μM from a 1000× stock in DMSO. γ irradiation was provided by a Gammacell 220 ^{60}Co source rated from 1-2 Gy/second during the course of these studies. Continuous γ irradiation was provided by a Gammacell 1000 ^{137}Cs source rated at 4 Gy/minute. UV irradiation at 254 nm was performed with a Stratalinker (Stratagene). Chromosome-spreading techniques were performed as described (Gasior et al., 1998). Scoring of spindle pole body duplication (SPD) in a strain expressing a *SPC42-GFP* gene fusion (Schutz and Winey, 1998) was by fluorescent microscopy (100×, Zeiss Axioskop) of formaldehyde fixed cells counting formation of a pair of adjacent or well-separated spots of green fluorescence. Elutriated G1 cells were obtained as described (Sidorova and Breeden, 1997).

Biochemistry

Protein was extracted by glass bead disruption in PBS pH 7.4, 5% glycerol, 0.5% Nonidet P40, 1 mM PMSF and protease inhibitor cocktail (Boehringer). For western analysis, 50 μg total protein was separated on 8% SDS-PAGE, transferred to nitrocellulose (Osmonics), probed with monoclonal α-Myc epitope antibody (9E10, Babco) and horseradish-peroxidase-conjugated sheep α-mouse secondary antibody (Amersham) and developed with Pierce SuperSignal using Hyperfilm ECL (Amersham). p13^{suc1}-(CalBiochem) associated histone H1 kinase assay was carreid out as described (Amon et al., 1992), using 50 μg protein/assay.

αf/nocodazole trap assay

5 ml of saturated overnight culture was diluted to ~10^7 cells/ml in 50 ml YPD with αf. Cells were incubated (~3 hours) at 30°C until >95% displayed mating projections. 10 ml aliquots were transferred to culture tubes, equilibrated on ice and irradiated with 0, 100, 200 or 400 Gy in the ^{60}Co irradiator. αf was removed by vacuum filtration with 100 ml water. Cells on filters were transferred to culture tubes and resuspended in 22°C YPD by vortexing. At 10 minute intervals, 0.5 ml aliquots were combined with 0.5 ml trapping media (15 μg/ml nocodazole, 15 μM αf in YPD), incubated for 90 minutes at 22°C then prepared for flow cytometry. A 2D gate (SSC versus FL2-A) on the basis of αf-arrested controls was used to determine the percentage of cells with 1N DNA content in test samples. 50,000 cells were analyzed from each sample.

Results

G1-arrested cells display physiological markers of the DNA damage response after γ irradiation

The mating pheromone alpha factor (αf) arrests haploid *MAT**a*** yeast cells in G1 with a 1N DNA content. We first investigated whether γ irradiation during αf arrest induces a DNA damage response by measuring the formation of repair foci containing the yeast RPA protein Rfa1p (Gasior et al., 1998; Wold, 1997). Brightly stained Rfa1 foci appeared on isolated chromosomes 15 minutes after 400 Gy γ irradiation but were completely absent from the chromosomes in unirradiated cells (Fig. 1A). Levels of Rfa1p remain constant after irradiation as shown by western analysis (Fig. 1B), indicating that the observed fluorescence is the result of protein localizing to the isolated chromosomes. Consistent with formation of repair foci, we observed a slower mobility of Rad53-13Myc (Sanchez et al., 1996) in irradiated αf-arrested cells dependent on DNA damage checkpoint sensors *RAD9* and *RAD17* (Fig. 1C).

S. cerevisiae maintains a 1N DNA content after γ irradiation

After release from αf, yeast enter S phase within 45 minutes

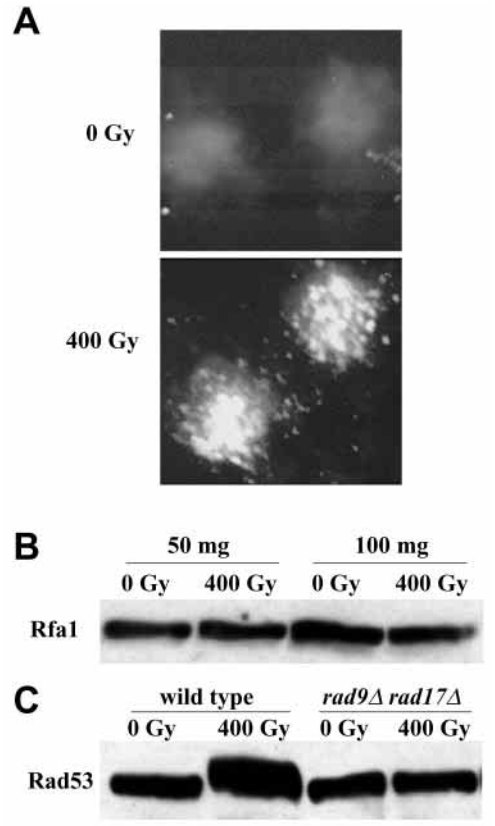

Fig. 1. G1 cells respond to γ radiation. Wild-type cells were αf arrested and irradiated or mock-treated then incubated at 22°C for 15 minutes before equilibrating on ice for processing. (A) Isolated chromosomes from αf-arrested cells were probed with rabbit α-Rfa1 (1:2000) then costained with DAPI and Alexa-488-conjugated α-rabbit secondary (1:400). The superimposition of DAPI and Alexa fluorescence is shown. (B) 50 μg protein from αf-arrested cells was run on 10% SDS-PAGE for western analysis. Blots were probed with α-Rfa1 (1:1000) and visualized as described in the Materials and Methods. (C) 50 μg protein from αf-arrested cells carrying *RAD53-13Myc* was run on 8% SDS-PAGE for western analysis.

and complete DNA synthesis by 90 minutes as monitored by the rise in DNA content (Fig. 2A). Prior exposure to γ irradiation results in a dose-dependent delay. Cells irradiated with 200 Gy (~10% lethality) begin to increase DNA content 30 minutes after unirradiated controls. After 1000 Gy (~70 DSB/haploid genome, ~95% lethality), cells remain 1N for over 2 hours (Fig. 2A).

These data are consistent with a regulated cell cycle delay before replication. An alternative hypothesis is that loss of viability and/or DNA damage itself may prevent timely replication. Nonetheless, both unirradiated and irradiated (1000 Gy; >99% lethality in checkpoint deficient strains) *rad17Δ* (Fig. 2B), *rad24Δ, rad17Δ rad24Δ rad9Δ* triple mutants and *mec1Δ* (not shown) mutants enter S phase within 30 minutes and continue replication with similar kinetics. This indicates that replication is possible, yet prevented by checkpoint activation. In contrast, cells only lacking *RAD9* remain 1N for over 2 hours after 1000 Gy irradiation (Fig. 1A). Since *rad9Δ* cells, unlike *rad17Δ* or *rad24Δ*, are proficient for the intra-S-phase checkpoint (Navas et al., 1996), the observed 1N delays cannot distinguish G1 arrest from stalled replication (Neecke et al., 1999).

In light of this result, it is of note that although increased γ irradiation dosage delays cell cycle progression in the early stages of the cell cycle, this does not correlate with rapid progression through mitosis. If G1/S DNA damage

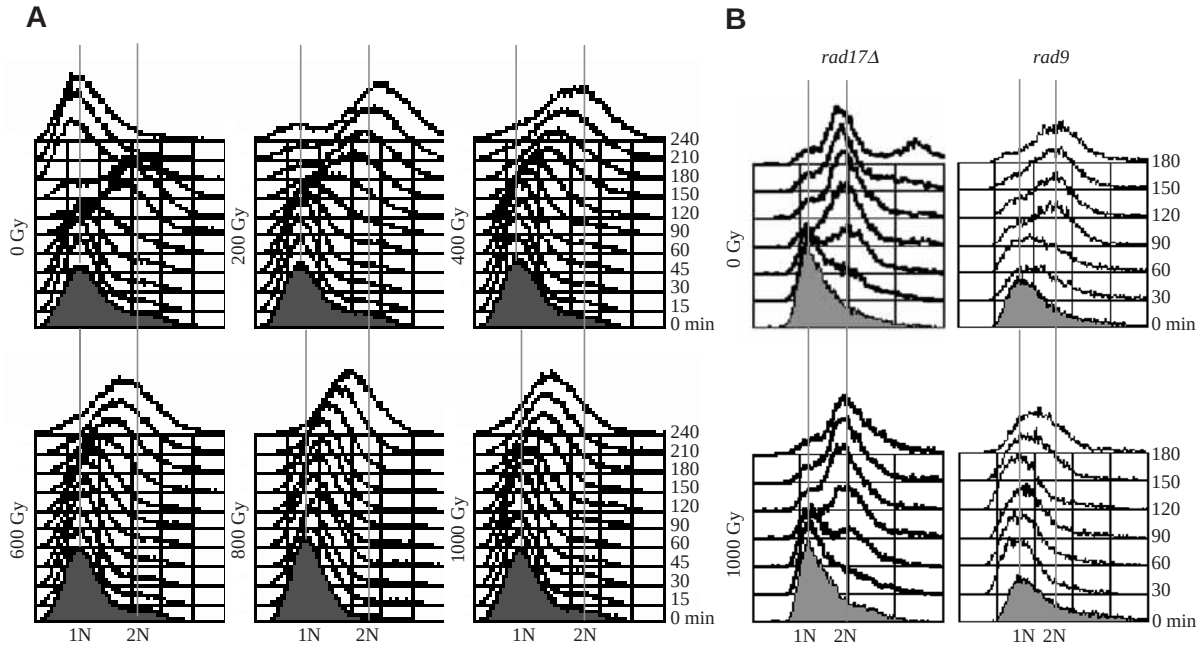

Fig. 2. G1 γ radiation results in dose-dependent replication delay. αf-arrested wild-type (A), *rad9Δ* and *rad17Δ* (B) cells were irradiated and released to media at 30°C. 0.5 ml aliquots were fixed at indicated time points for flow cytometry. Lines drawn across graphs show 1N and 2N positions. Identical results were obtained with ρ^0 cells (data not shown).

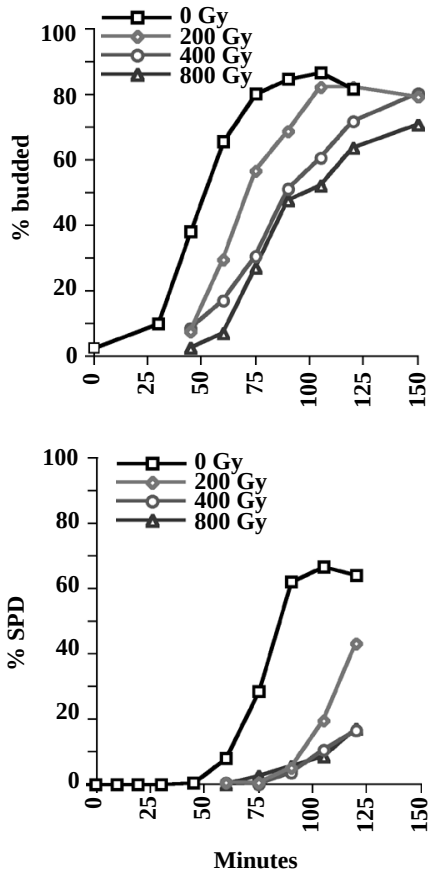

Fig. 3. Spindle pole body duplication and bud emergence delay after G1 γ irradiation. *SPC42-GFP* (Schutz and Winey, 1998) strains were arrested in αf and released following irradiation. 0.5 ml aliquots of cells were fixed in 3.7% formaldehyde to score bud emergence and SPD or fixed for flow cytometry (data not shown).

checkpoints allowed for completion of DNA repair, then it might be expected that cells would not delay in a subsequent mitosis. Unirradiated cells reach a 2N DNA content at 120 minutes after release from αf (Fig. 2A). A return to 1N DNA content is observed at 150 minutes, and the majority of cells are 1N at 180 minutes. Mitosis, defined in this assay as the length of time spent at 2N, lasted 1 hour in unirradiated cells. After 200 Gy, which delayed replication by 30 minutes, cells remained at 2N until 240 minutes after αf release. In other experiments, this 2N delay after 200 Gy was found to persist for at least 5 hours (data not shown). Similarly, no return to 1N DNA content was observed at higher γ irradiation doses. This again could argue for a lack of G1 DNA damage checkpoint regulation or for an adaptive response to γ irradiation in G1.

Early G1/S events are delayed after DNA damage

Although progression and completion of DNA synthesis are subject to intra-S-phase checkpoint regulation (Paulovich and Hartwell, 1995), completion of other Start events remains unaffected by stalled replication (e.g. hydroxyurea treatment). We examined bud formation and spindle pole body duplication (SPD) after G1 irradiation in cells carrying fluorescently marked spindle poles via expression of a *SPC42-GFP* gene fusion (Schutz and Winey, 1998) (Fig. 3). In unirradiated cells, DNA synthesis, as measured by flow cytometry, is >50% complete by 20 minutes. Bud emergence is ~50% complete by 40 minutes and SPD is ~50% complete by 75 minutes. After 200 Gy, 50% budding is only reached after 60 minutes, whereas SPD is 50% complete at ~120 minutes after release. These delays increase with dose increases. After 400 Gy and 800 Gy, 50% budding is reached at 90 minutes, whereas >25% SPD is not reached during the 120 minute time course.

We also assayed the appearance of active CDK as an independent marker for passage through Start. We used the *RAD53-13Myc*-expressing strain to simultaneously report DNA damage signaling (Fig. 4A,B). After release from αf, Cdc28 kinase activity is detected at 30 minutes in unirradiated wild-type cells, and Rad53-13Myc remains unshifted (Fig. 4A). However, 200 Gy delayed appearance of Cdc28p kinase activity to ~45 minutes by which time Rad53-13Myc had returned to its unshifted mobility. 400 Gy induced a persistent shift of Rad53-13Myc and Cdc28 kinase activity did not appear

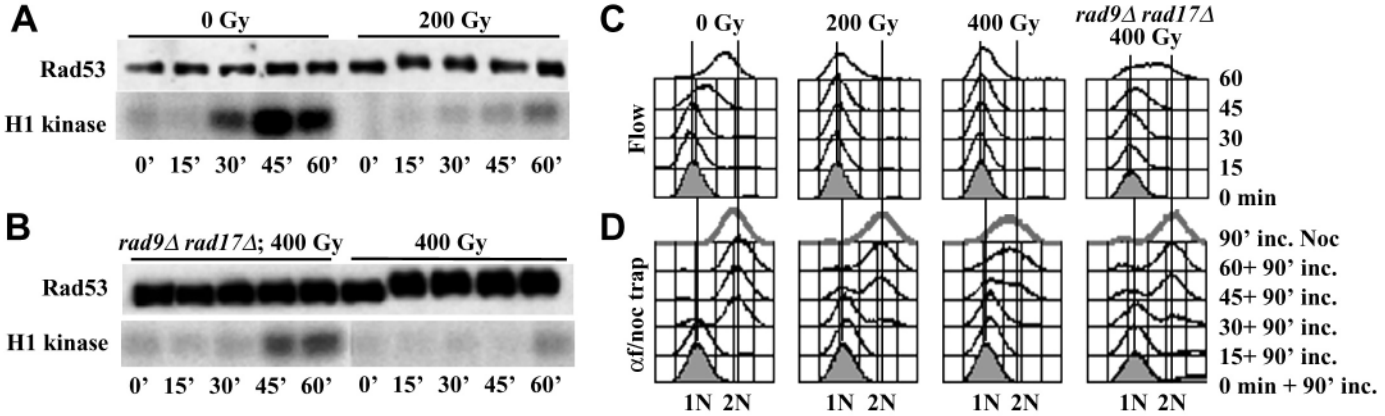

Fig. 4. Rad53-13Myc shifts while G1 is extended. *RAD53-13Myc* cells were arrested with αf and released following irradiation. Protein samples were collected for α-Myc westerns, and $p13^{suc1}$ associated histone H1 kinase activity (A,B) was measured at the indicated times. At the same time points, additional aliquots were fixed for flow cytometry (C) or incubated for 90 minutes in trapping media (with and without αf) before fixation (D). The slight delay in *rad9Δ, rad17Δ* progression after irradiation compared with unirradiated wild type was observed only in the *RAD53-13Myc* background.

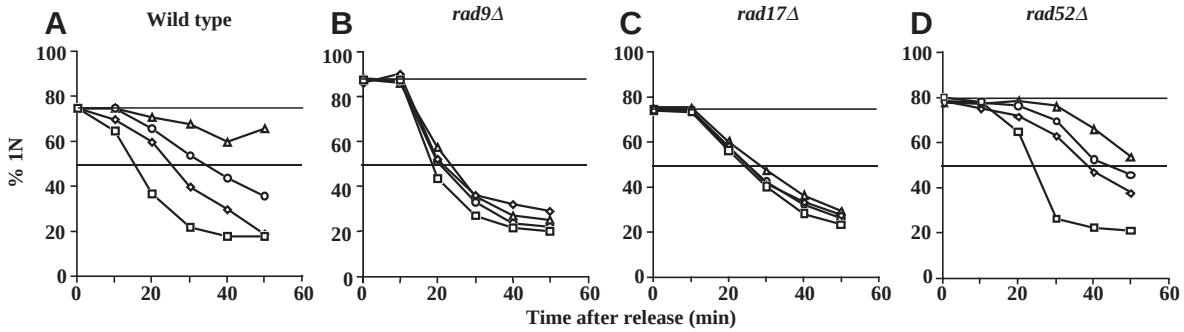

Fig. 5. Genetic requirements for G1 arrest. αf-arrested cells were released after increasing doses of γ irradiation. αf/nocodazole-trapping media was added at the indicated time points, and cells were incubated 90 minutes before fixation. (For wt, *rad9Δ* and *rad17Δ*: □, 0 Gy; ◇, 100 Gy; ○, 200 Gy; △, 400 Gy. For *rad52Δ* (□, 0 Gy; ◇, 50 Gy; ○, 100 Gy; △, 200 Gy)). For comparisons, the solid line shows the percentage of 1N of the starting population and the hatched line marks 50% 1N. *rad9Δ, rad17Δ* double mutants were indistinguishable from either single mutant in this assay (data not shown).

until 60 minutes. For the *rad9Δ rad17Δ RAD53-13Myc* double mutant, 400 Gy did not delay Cdc28 kinase activity nor shift Rad53-13Myc (Fig. 4B).

Delayed budding, SPB duplication and Cdc28 activity all suggest G1 arrest, but they are not positive indicators. By contrast, yeast cells are only responsive to mating factor in G1. After release from αf, cells remain sensitive to re-arrest by αf for less than 30 minutes. After that, they pass Start and become αf resistant. We tested whether DNA damage in G1 can extend this window of αf sensitivity. During the time course in Fig. 4A,B, additional aliquots were fixed for flow cytometry. Irradiated cells appeared to delay replication after release from αf (Fig. 4C). To determine whether these cells remained in G1 or had entered S phase, aliquots were incubated for 90 minutes in αf and nocodazole (Fig. 4D). The subsequent addition of αf arrested G1 cells with a 1N DNA content, whereas nocodazole trapped cells that had passed Start (Siede et al., 1996). Unirradiated *RAD53-13Myc* cells are no longer arrested by αf at 30 minutes and accumulate with 2N DNA content. 30 minutes after 200 Gy, αf continues to arrest >80% of cells. After 400 Gy, ~60% of cells can be arrested by αf at 45 minutes. However, in the *rad9Δ rad17Δ RAD53-13Myc* double mutant irradiated with 400 Gy, cells lost αf resistance by 45 minutes. Nonetheless, all cells were capable of increasing DNA content. When incubated in nocodazole alone at the 0 minutes timepoint, both the irradiated and unirradiated cells accumulate at 2N by 90 minutes (Fig. 4D, top histograms).

Genetic characterization of γ-irradiation-induced G1 arrest

We used a quantitative version of this αf/nocodazole trap assay to examine genetic regulation of the G1 arrest. Unirradiated wild-type, *rad9Δ* and *rad17Δ* cells rapidly lose αf sensitivity, as less than 50% of cells remain in G1 20 minutes after release (Fig. 5). Wild-type cells display a γ radiation dose-dependent delay in exit from G1 (Fig. 5A), whereas 50% of *rad9Δ* (Fig. 5B) and *rad17Δ* (Fig. 5C) mutants remain in G1 at 20 minutes regardless of the dose. Mutants lacking *RAD52* are defective in both recombinational and end-joining DSB repair (Hegde and Klein, 2000). Treated with only 50 Gy, *rad52Δ* mutants maintain a 50% G1 population 18 minutes longer than unirradiated cells, which is proportional to the 200 Gy delay in repair-proficient wild-type cells (Fig. 5D).

The previous experiments establish a G1 arrest but can not rule out the possibility that replication checkpoints play a role in the observed delays. *POLε* detects DNA damage encountered by the replication fork and is required for the intra-S phase checkpoint (Navas et al., 1995). The αf/nocodazole trap assay demonstrates that checkpoint defective *polε-12::kan*^{R} cells irradiated with 200 Gy remain sensitive to αf at 50 minutes after release, whereas unirradiated controls and irradiated or unirradiated *rad9Δ polε-12::kan*^{R} double mutants become αf resistant after 20 minutes (Fig. 6).

The G1 checkpoint in continuous irradiation

The previous results used γ radiation doses that induce 10-100 DSB per haploid yeast genome in 2-20 minutes, inducing 10-95% lethality (Frankenberg-Schwager and Frankenberg, 1990). At these high doses, checkpoint responses might be complicated by stress and survival responses. To eliminate

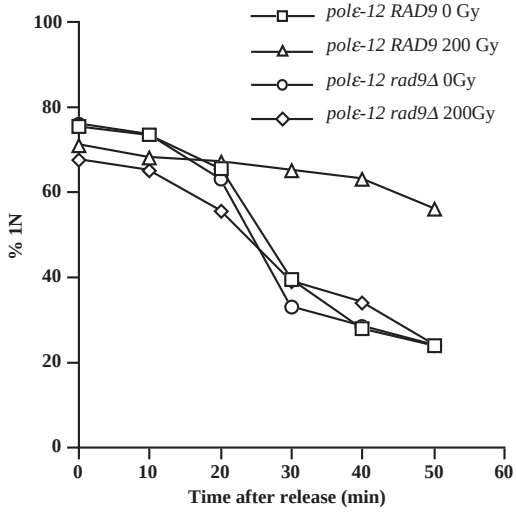

Fig. 6. *POLε* is not required for the G1 checkpoint. Indicated strains were treated as in Fig. 5.

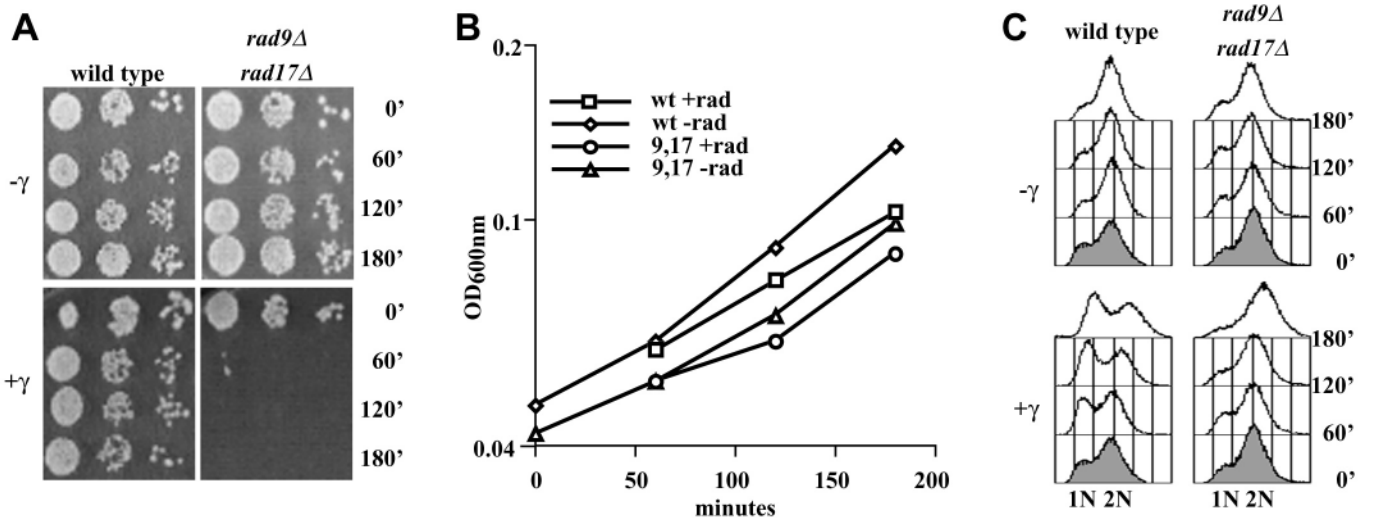

Fig. 7. G1 delay is the predominant response to tolerable γ radiation. Log phase cells were incubated for 3 hours at 450 rad/minute. At 60 minute intervals, cells were collected for plating efficiency (A), OD_{600} measurements (B) or fixed for flow cytometry (C). In (A), 3 μl of 10-fold serial diluted cells were spotted to YPD plates and incubated 48 hours at 30°C.

these effects and assess checkpoint regulation at a tolerable radiation dose, we incubated cells in rich media at 22°C in a ^{137}Cs irradiator that induces ~0.25 DSB/minute. Wild-type log-phase cells irradiated for 4 hours delay cell division but remain viable as measured by plating efficiency (Fig. 7A,B). *rad9Δ rad17Δ* double mutants show only slight delays in growth and rapidly lose viability. Flow cytometry of the irradiated wild-type cells revealed that the 1N population of cells steadily increases for 1 hour (Fig. 7C). In turn, the proportion of cells that have passed Start and display small to medium sized buds decreases from 33% to 12% within 30 minutes. No G1 shift is observed in the irradiated *rad9Δ rad17Δ* cells.

To test if this tolerable radiation maintains G1, αf arrested cells were released and incubated in the ^{137}Cs irradiator for 90 minutes. Without irradiation, cells enter the cell cycle promptly and complete replication by 60 minutes (Fig. 8A). Irradiated cells remained unbudded, with 1N DNA content even after 90 minutes. However, once removed from the irradiator, these cells began replication without delay (Fig. 8B). Confirming a G1 arrest, αf restricted 57% of these cells from completing S phase (Fig. 8C). Despite being arrested in G1, the irradiated cells do not lose viability (not shown). This G1 arrest was also observed when the synchronized G1 population was obtained through elutriation (not shown).

These data also provide a comparison with the results in Fig. 2. 90 minutes in the ^{137}Cs source results in 450 Gy accumulated γ irradiation. In Fig. 2A, it was found that cells receiving a similar dose, delivered in about 7 minutes, maintained a 1N DNA content 60 minutes longer than unirradiated controls (400 Gy panel). In contrast, cells removed from the ^{137}Cs source increased DNA content without delay as though no accumulated damage was retained. This argues for ongoing repair in G1 during the ^{137}Cs treatment, although these results do not exclude the possibility that less strand breaks occur in the low-level treatment.

G1 arrest can be maintained for over 18 hours

The G1 delay and replication checkpoints are described as transient delays before a persistent G2/M arrest. Asynchronous wild-type cells in liquid media treated with 3000 Gy, the minimum dose for 0% viability, were plated at low dilution onto YPD to monitor bud emergence and division of individual cells (Fig. 9A). Of the 38±5% of unbudded cells at 0 hours, 23±4% remained unbudded at 18 hours. In a *rad9Δ rad17Δ* double mutant, the unbudded cells dropped from 43±4% to 9±3% after 18 hours. This loss was reflected in 48±3% of cells undergoing second and third divisions. Alternatively, cells

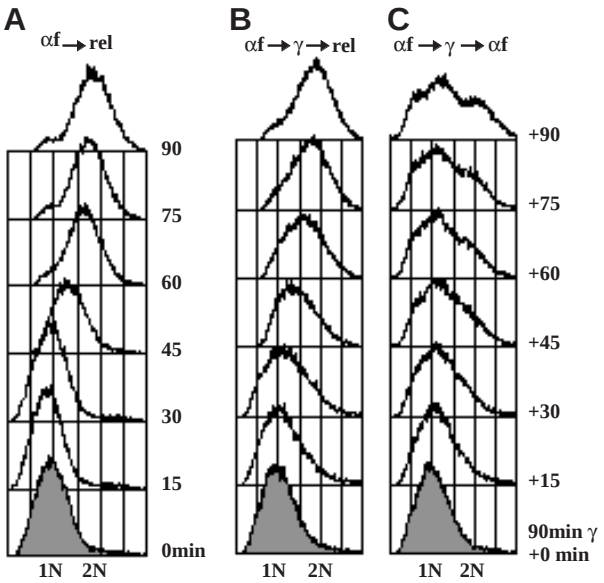

Fig. 8. Tolerable γ radiation maintains G1. αf-arrested cells were released into fresh media. Cells were split between a 22°C control (A) and irradiated samples (B) and (C). 15 minute time points were then collected from controls for flow cytometry. 90 minutes after release, irradiated samples were removed from the ^{137}Cs source and incubated with (C) or without (B) αf, and the time course was repeated.

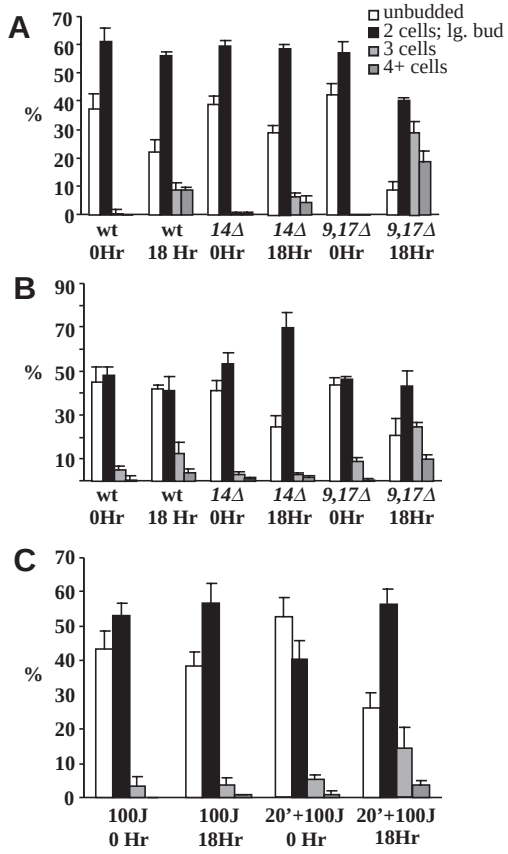

Fig. 9. Unbudded G1 arrest can last for 18 hours after DNA damage. Log-phase strains were irradiated with 3000 Gy then diluted to ~10^4 cells/ml before plating on YPD (A) or were plated before UV irradiation at 100 J/m^2 (B). Cell numbers were counted immediately (0 Hr) and at 18 hours post irradiation. (C) Cells were plated and incubated 0 or 20 minutes before 100 J/m^2 irradiation. (Error bars represent s.d. of three experiments.)

were first plated then exposed to 100 J/m^2 UV irradiation, a lethal dose. Here, the unbudded population in wild-type cells only dropped from 45±7% to 42±2% after 18 hours (Fig. 9B), whereas the unbudded population in *rad9Δ rad17Δ* cells dropped from 44±3% to 21±7%. Even after 18 hours unbudded cells were both alive and remained in G1 (Schrick et al., 1997). Over 30% of wild-type cells from the above experiments formed mating projections after 3.5 hours incubation in αf after either γ (Fig. 10A) or UV irradiation (Fig. 10B).

UV radiation of a strain lacking the *RAD14* excision repair gene resulted in a drop in unbudded cells from 42±4% to 25±6% at 18 hours (Fig. 9B). Unlike *rad9Δ rad17Δ*, these cells accumulated with large buds, suggesting that the G1 checkpoint alone is compromised in the *rad14Δ* background. By contrast, γ irradiation of the *rad14Δ* strain yielded a G1 arrest indistinguishable from wild type (Fig. 9A).

The 50 minutes required to deliver 3000 Gy, compared with <10 seconds for 100 J/m^2, may underlie the apparent superiority of UV irradiation in maintaining unbudded arrest. When wild-type cells were incubated for 20 minutes on plates before UV treatment (Fig. 9C), 53±5% of the cells remained unbudded immediately after irradiation. However, only 26±5% remained unbudded after 18 hours.

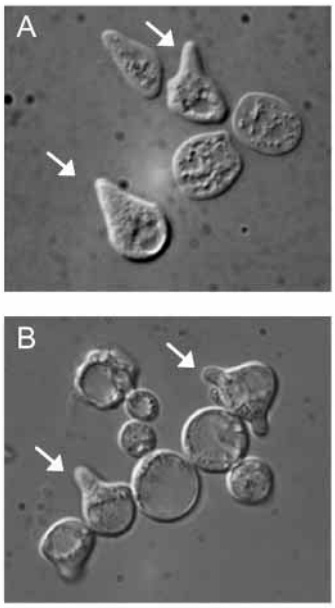

Fig. 10. Unbudded cells remain sensitive to αf. Wild-type cells (see Fig. 9A,B) were collected 18 hours after γ (A) or UV (B) radiation and incubated in αf for 3.5 hours at 30°C. Cells were fixed as for flow cytometry and scored (X63, Zeiss Axioskop) for formation of mating projections (arrow). Mating projections were not observed after incubation without αf.

Discussion

Although many eukaryotes arrest in G1 after DNA damage, haploid budding yeast perform a *RAD9*-dependent G2/M arrest. A common rationale is that DSB repair in G1 is futile without the homologous sister chromosomes needed for efficient repair. Yet, DNA damage in haploid G1 yeast (1) activates Rad53 (Sidorova and Breeden, 1997), (2) is processed (de la Torre-Ruiz and Lowndes, 2000) and (3) promotes a transient G1 delay (Siede et al., 1993). This argues for a G1 checkpoint even if it is simply en route to a mitotic arrest. Nonetheless, a recent report (Neecke et al., 1999) has challenged even the existence of G1 arrest in yeast, proposing that the delay depends on the intra-S-phase checkpoint. Replication may provide substrate for homologous recombination in DSB repair, yet replication also dissociates damaged DNA from the repair template. So under what circumstances might the cell rely on G1 arrest rather than S-phase or G2/M checkpoints? We have re-examined the budding yeast response to γ and UV irradiation in G1. We establish a bona fide G1 DNA damage checkpoint in haploid yeast cells and suggest that, in some circumstances, this response is more pronounced than intra-S phase or G2/M checkpoints. Our criteria for an authentic G1 DNA damage checkpoint are that the cell must detect damage prior to replication and signal an arrest that prevents the cell from passing Start. To be physiologically significant, the duration of arrest should be dose dependent and reflect the cells' capacity to repair lesions.

The recruitment of Rfa1p to damage foci and transient activation of Rad53p demonstrate that G1 haploid yeast detect strand breaks and signal their presence. We then used several criteria to determine the phase of the ensuing arrest. Spindle pole duplication, bud emergence and activation of Cdc28 are

all delayed in a dose-dependent manner, which strongly argues for prolonged G1. Finally, the αf/nocodazole trap assay quantitatively determined the proportion of G1 cells during a DNA damage response. We demonstrated that wild-type cells remain αf sensitive after γ irradiation and that prolonged αf sensitivity absolutely depends on checkpoint signaling. *RAD17*, *RAD24* or *MEC1* mutants become rapidly αf resistant irrespective of the dose. Distinguishing this checkpoint from the intra-S-phase delay, mutation of *RAD9* completely abrogates G1 arrest. In turn, G1 arrest is unaffected by a *POLε* mutation that voids the replication checkpoint.

A key question is whether there is a link between repair and recovery from the G1 checkpoint. Clearly, the delay is proportional not only to the amount of damage but also to the extent of repair. In haploid cells, G1 arrest is prolonged by mutating *RAD52* or *YKU70*, genes involved in non-homologous end-joining repair. Strikingly, diploid yeast do not arrest in G1 after 400 Gy, unless they lack recombinational repair genes *RAD50* or *RAD57* (J.N.F.G. and S.J.K., unpublished). Increased delay in DSB repair mutants suggests that the G1 arrest allows significant repair and prevents a potentially deleterious S phase and mitosis. However, it is not certain whether the G1 cell is capable of complete repair before passing Start or even whether cells in G1 are responsive only to certain types of lesions.

After 200 Gy, the phosphorylated form of Rad53p is lost as cells pass Start. At 400 Gy, Start occurred even in the presence of phosphorylated Rad53p. One interpretation is that competing pathways may promote recovery and cell cycle progression. Cells may wait for repair to be completed but adapt and proceed if repair is not possible. Alternatively, G1 cells may repair lesions that would be deleterious to replication yet pass Start if the remaining damage is better suited for replicative repair or homologous recombinational repair. Indeed, it was found that a 200 Gy dose of γ irradiation arrests cells in G1 for 15 minutes, but these same cells progress to G2/M and remain with a 2N DNA content for several hours after unirradiated cells have divided and returned to G1. This is consistent with unrepaired lesions remaining after the G1 checkpoint.

Evidence that the G1 arrest does facilitate timely repair and therefore protects viability was found when cells were incubated in a tolerable low-level γ source. In these experiments, wild-type cells accumulate in G1 and are able to survive a 3 hour exposure. Checkpoint mutants fail to arrest and rapidly lose viability. Importantly, wild-type G1 cells released from the ^{137}Cs source initiate and complete replication as rapidly as unirradiated cells. If ongoing repair had not occurred in G1, damage would be expected to accumulate, leading to low viability and delayed replication, as was found when similar doses were delivered in the ^{60}Co source. In fact, S phase only delayed when the G1 checkpoint was compromised in *rad9*Δ mutants.

The duration of γ- and UV-induced arrest differs in repair-deficient backgrounds. UV irradiation was clearly capable of inducing a G1 arrest, but this was compromised in the absence of *RAD14*. Yet, in response to γ irradiation, *rad14*Δ strains arrest in G1, as do mismatch repair mutants (not shown). This argues that UV lesions, unlike γ-radiation-induced DSBs, may require DNA metabolism to promote checkpoint signaling. The excision activity of Rad14p may be a limiting factor for the conversion of thymidine dimers and other UV damage to the single-stranded gaps that activate checkpoint proteins. In cells lacking Rad14p, Polε-regulated stalling of the replication fork provides an alternate, Rad9p-independent checkpoint mechanism. This is a very attractive answer to the paradoxical results of Neecke et al. (Neecke et al., 1999).

In conclusion, our results substantiate an authentic G1 checkpoint arrest in budding yeast that is dependent on the amount of DNA damage and the extent of repair. This arrest is physiological, depends on checkpoint signaling pathways and appears to be a primary response to low-level, tolerable γ radiation and is a terminal arrest point after lethal UV or γ irradiation.

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