The G1-S checkpoint in fission yeast is not a general DNA damage checkpoint

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Accepted 10 September 2008
Journal of Cell Science 121, 4047-4054 Published by The Company of Biologists 2008
doi:10.1242/jcs.035428

Summary

Inhibitory mechanisms called checkpoints regulate progression of the cell cycle in the presence of DNA damage or when a previous cell-cycle event is not finished. In fission yeast exposed to ultraviolet light the G1-S transition is regulated by a novel checkpoint that depends on the Gcn2 kinase. The molecular mechanisms involved in checkpoint induction and maintenance are not known. Here we characterise the checkpoint further by exposing the cells to a variety of DNA-damaging agents. Exposure to methyl methane sulphonate and hydrogen peroxide induce phosphorylation of eIF2α, a known Gcn2 target, and an arrest in G1 phase. By contrast, exposure to psoralen plus long-wavelength ultraviolet light, inducing DNA adducts and crosslinks, or to ionizing radiation induce neither eIF2α phosphorylation nor a cell-cycle delay. We conclude that the G1-S checkpoint is not a general DNA-damage checkpoint, in contrast to the one operating at the G2-M transition. The tight correlation between eIF2α phosphorylation and the presence of a G1-phase delay suggests that eIF2α phosphorylation is required for checkpoint induction. The implications for checkpoint signalling are discussed.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/121/24/4047/DC1

Key words: Checkpoint, DNA replication, Cell cycle, DNA damage, G1 phase, eIF2α, Fission yeast

Introduction

Cell proliferation demands that the cells go through the mitotic cell cycle, involving duplication of their DNA before chromosome segregation and cell division. During G1 phase the decision is made whether to start a new round of the cell cycle, go into a quiescent state or enter into meiosis. It is critical for the cells to carefully regulate the progression through G1 phase, where the cells become committed to a new round in the cell cycle. Cell cycle progression is negatively regulated by checkpoint mechanisms to make sure that the events of one cell cycle phase have been completed before continuing to the next phase. Checkpoints delay the cell cycle in response to several forms of stress. DNA damage checkpoints are thought to allow additional time for DNA repair before DNA replication (S phase) and before chromosome segregation (mitosis). These responses are crucial for the cells to maintain their genetic integrity. The fact that the majority of cancer cells have defects in G1-S checkpoints (Bartek et al., 2004; Nojima, 2004; Sherr and McCormick, 2002) underlines their importance.

Several DNA damage checkpoints have been described in the model organism *Schizosaccharomyces pombe* (Carr, 2002; Cuddihy and O'Connell, 2003; Huberman, 1999): The intra-S checkpoint slows down DNA replication in the presence of DNA damage. The S-M checkpoint monitors the replicative status of the DNA and prevents mitosis when replication is incomplete. The G2-M checkpoint prevents transition from G2 into mitosis in the presence of DNA damage. Finally, the G1-M checkpoint prevents mitosis in cells that are arrested in G1 phase. These checkpoints are all dependent on the checkpoint Rad proteins and on one or both of the protein kinases Cds1 and Chk1 that inhibit Cdc2 activity by promoting its phosphorylation. We have recently described a novel checkpoint in *S. pombe* regulating the G1-S transition (Nilssen et al., 2003; Tvegård et al., 2007), where G1 cells are delayed in S-phase entry after exposure to ultraviolet light (254 nm; UVC). In contrast to the other checkpoints listed above the G1-S checkpoint acts independently of Cds1 and Chk1 and does not lead to Cdc2 phosphorylation. The checkpoint is totally dependent on the Gcn2 kinase, which is best known for its role in the response to amino acid depletion in budding yeast. Gcn2 is a regulator of translation and, consistently, checkpoint activation is accompanied by a marked reduction in the rate of general translation.

Different forms of stress cause cells to alter their pattern of transcription and translation to adapt to changes in the physiological conditions. Stress-induced regulation of translation occurs mostly at the level of initiation and involves the eukaryotic translation initiation factor 2 (eIF2). It is conserved from yeast to mammals and has a key role in regulating mRNA translation. eIF2 is responsible for delivering the charged methionyl-tRNA to the ribosome to initiate translation. Phosphorylation of the α-subunit of eIF2 at serine 51 (Ser52 in *S. pombe*) reduces the activity of the eIF2 complex, resulting in a rapid decrease in global translation rate. eIF2α is phosphorylated in response to a wide range of environmental stress conditions and in many organisms (Fels and Koumenis, 2006; Hinnebusch, 2000; Wek et al., 2006). In *S. pombe* eIF2α can be phosphorylated by either of the three kinases Gcn2, Hri1 and Hri2, which are activated in different stress situations (Zhan et al., 2004).

The UVC-induced G1-S checkpoint is totally dependent on the Gcn2 kinase (Tvegård et al., 2007), but the mechanism of its activation and its role in the checkpoint are not known. In particular, the role of eIF2α phosphorylation and translational downregulation in the checkpoint is not clear. To better understand the mechanistic connection between cellular stress, phosphorylation of eIF2α and checkpoint activation, we exposed *S. pombe* cells to different kinds of stress and monitored phosphorylation of eIF2α and cell cycle

progression at G1-S. We found a tight correlation between eIF2α phosphorylation and checkpoint induction. Some but not all of the DNA-damaging agents failed to induce both eIF2α phosphorylation and a G1 delay, showing that the G1-S checkpoint is not responding to DNA damage in general.

Results

UVC induces strong eIF2α phosphorylation

After UVC irradiation of *S. pombe* cells in G1 phase eIF2α becomes rapidly phosphorylated at serine 52 and, at the same time, the cells are delayed in their entry into S phase (Tvegård et al., 2007). Here we investigate the UVC-induced eIF2α phosphorylation and the cell-cycle delay in more detail. Phosphorylation was induced in a dose-dependent manner in cells synchronised in G1 (Fig. 1A) as well as in asynchronous cells (data not shown). To evaluate the extent of UVC-induced eIF2α phosphorylation we compared it to that observed after amino acid starvation, a treatment known to activate Gcn2 and eIF2α phosphorylation both in yeasts and mammalian cells (Dever and Hinnebusch, 2005; Zhan et al., 2004). UVC irradiation with a dose giving a cell survival of ~15% resulted in a similar level of eIF2α phosphorylation than that found after a 2-hour essential amino acid starvation (Fig. 1B). These data strongly suggest that the phosphorylation induced by the UVC dose used is significant and can be expected to have dramatic physiological consequences.

To investigate whether the UVC-induced eIF2α phosphorylation is specific for G1 phase we exposed cells synchronised in either G1, S or G2 phase to a constant dose of UVC. The level of eIF2α phosphorylation was similar for cells in all cell cycle phases (Fig. 1C), demonstrating that it is not specific for G1 phase, but occurs in all cell cycle phases. Whether eIF2α phosphorylation or the resulting reduction of translation affect cell cycle progression outside G1 phase have not been investigated. When activated, the S-M and G2-M checkpoints impose significant delays, which may explain why a possible additional delay caused by translational downregulation has remained unnoticed.

The level of eIF2α phosphorylation decreased only slowly after its induction by UVC in G1 phase. A time-course study revealed that the elevated phosphorylation level persisted for about 90 minutes after irradiation (Fig. 1D), concurring with the time of S-phase entry (Tvegård et al., 2007).

Hydrogen peroxide induces eIF2α phosphorylation and a cell-cycle delay

Exposing asynchronous wild-type cells to increasing doses of hydrogen peroxide (H_2O_2) resulted in a steadily increasing level of eIF2α phosphorylation (Fig. 2A). The phosphorylation induced by 5 mM H_2O_2 was largely maintained for up to 2 hours after exposure in wild-type G1 cells (Fig. 2B). Hardly any phosphorylation was detected in the *gcn2* mutant (Fig. 2C) although some phosphorylation was apparent at the 120-minute time point. This phosphorylation is probably due to the Hri2 kinase, which is known to become activated in response to H_2O_2 treatment (Dunand-Sauthier et al., 2005; Zhan et al., 2004).

To examine the cell-cycle progression in response to H_2O_2, G1-synchronised cells were initially analysed by flow cytometry. Both the untreated control and H_2O_2-treated cells contained one copy of the genome (1C) at time 0, reflected in the presence of one major peak in the DNA histograms (Fig. 2D). Most of the unexposed control cells had entered S phase by 60 minutes, when most of the cells contained between 1C and 2C of DNA. Both wild-type and *gcn2* cells delayed the increase in DNA content from 1C to 2C in response to H_2O_2 treatment (Fig. 2D), reflecting a cell cycle delay. The flow cytometry results do not tell us exactly where in the cell cycle the delay occurs, but only that they do not go far into S phase. To investigate more closely whether the delay occurred prior to or after entry into S phase we measured chromatin loading of the pre-replicative complex (pre-RC) (Kearsey et al., 2000). Pre-RC formation, an obligatory step in preparation for S phase, involves the loading of the Mcm complex onto chromatin, an association that lasts until the end of S phase. The kinetics of pre-RC formation was measured by detecting chromatin-bound, GFP-tagged Mcm6 (Kearsey et al., 2000). At 60 minutes the majority of the untreated wild-type cells had loaded Mcm6 onto chromatin (Fig. 2E, left panel), in agreement with the flow cytometry data. However, Mcm6 loading was dramatically delayed in cells treated with H_2O_2, showing that the cell cycle of wild-type cells is delayed in G1 phase by H_2O_2. By contrast, the treated and untreated *gcn2* mutant cells

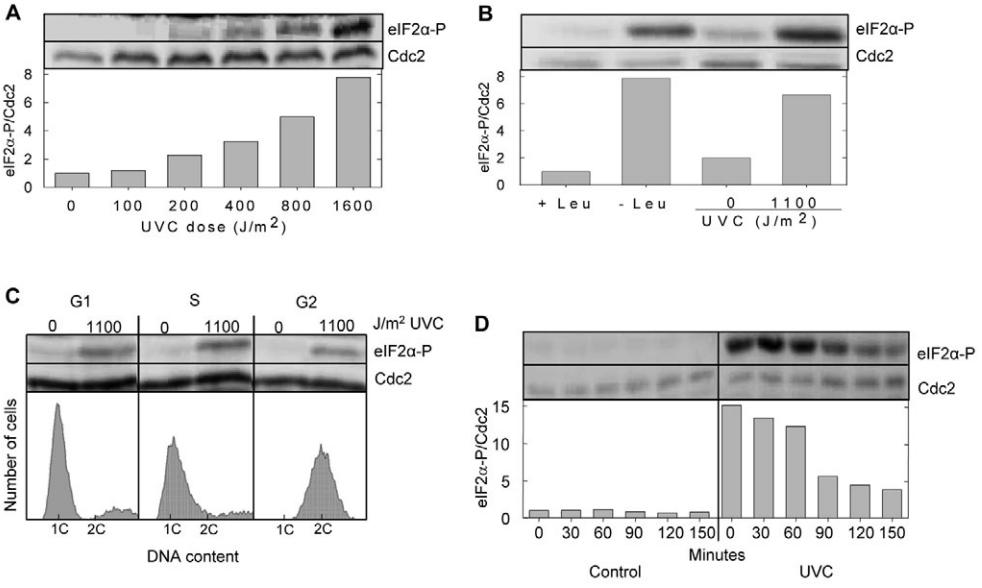

Fig. 1. UVC-induced phosphorylation of eIF2α. Immunoblots of total cell extracts probed with antibody against eIF2α-P and against Cdc2 as loading control, and the quantification (bottom). (A) G1-synchronised *cdc10-M17* cells exposed to increasing doses of UVC irradiation. (B) Asynchronous cells auxotrophic for leucine, which were starved of leucine for 2 hours (–Leu) and asynchronous wild-type cells exposed to 0 or 1100 J/m^2 of UVC. (C) Synchronous *cdc10-M17* cells in G1, S and G2 phase exposed to 0 and 1100 J/m^2 of UVC. The corresponding DNA histograms are shown below. (D) G1-synchronised *cdc10-M17* cells irradiated with 0 (control) or 1100 J/m^2 of UVC and incubated for different times after irradiation, as indicated.

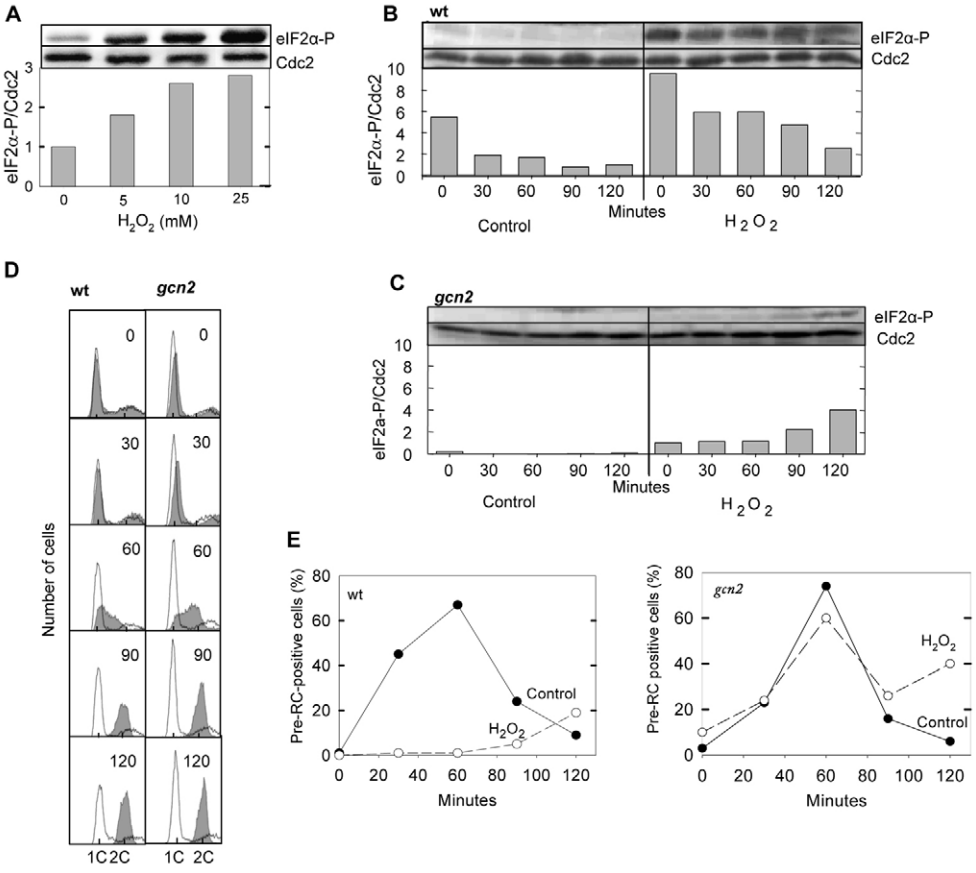

Fig. 2. H_2O_2-induced eIF2α phosphorylation and cell cycle delay. (A) Immunoblot (see Fig. 1 for details) of asynchronous cells exposed to increasing concentrations of H_2O_2. G1-synchronised wild-type (B) and *gcn2* mutant (C) cells exposed to 0 (control) or 5 mM H_2O_2 and incubated for different times, as indicated. The quantified relative value for H_2O_2 at time 0, was arbitrarily set to 1. (D) DNA histograms of G1-synchronised wild-type (left) and *gcn2* (right) cells that were untreated (control; shaded histogram) or treated with 5 mM of H_2O_2 (unshaded) and incubated for the times indicated (in minutes) after H_2O_2 treatment. (E) Pre-RC assays of G1-synchronised wild-type (left) or *gcn2* mutant (right) cells after exposure to 0 (control) or 5 mM of H_2O_2 and incubated for different periods of time, as indicated.

loaded pre-RCs with the same kinetics (Fig. 2E, right panel), further showing that the induced G1 delay is dependent upon Gcn2. When the *gcn2* cells enter into S phase without delaying, in response to H_2O_2, it is probable that they do so with DNA damage present. Consistently, a large percentage of the *gcn2* cells maintain Mcms in a chromatin-bound form even 120 minutes after exposure, indicating that DNA replication is not finished. We conclude that H_2O_2 induces a Gcn2-dependent delay in progression from G1 to S phase.

MMS induces eIF2α phosphorylation and a cell-cycle delay

Asynchronously growing cells were treated with different concentrations of the alkylating agent methyl methane sulfonate (MMS) for 1 hour, followed by MMS inactivation and incubation for 1 hour before samples were collected. Exposure to MMS resulted in the phosphorylation of eIF2α in a concentration-dependent manner (Fig. 3A), at concentrations giving a cell survival below 25% (Fig. 3B). In order to investigate the effect on cell cycle progression, G1-phase cells were treated with 0.15% MMS for 15 minutes (5% cell survival) and washed prior to sample collection to inactivate and remove the MMS. A strong increase in phosphorylation of eIF2α was also observed in these cells and the phosphorylation response remained even 2 hours after MMS had been removed (Fig. 3C). In the *gcn2* mutant a weak phosphorylation response was seen immediately after MMS treatment, but was not detected 30 minutes later (Fig. 3D). This transient phosphorylation was shown, in separate experiments, to be induced in a Gcn2-independent manner by the centrifugation involved in washing the cells (supplementary material Fig. S1).

The cell-cycle progression of G1 cells exposed to MMS was analyzed by flow cytometry. The unexposed cells, both wild type and *gcn2*, increased their DNA content from 1C to 2C between 60 and 90 minutes after exposure, in agreement with previous results (Fig. 2D) (Tvegård et al., 2007). After MMS treatment both wild-type and *gcn2* cells delay cell-cycle progression with a 1C DNA content (Fig. 4A). The DNA histogram peaks become broader at 120 minutes, suggesting that the cells may have entered S phase by this time, but neither of the two strains completed DNA replication by 150 minutes (Fig. 4A). These observations show that MMS-treated wild-type and *gcn2* cells delay progression through S phase and possibly also entry into S phase.

To explore whether entry into or through S phase is delayed we ideally wanted to measure the timing of pre-RC loading after MMS treatment as above, but decided to employ another assay, for the following reason. The inactivation and removal of MMS was time-consuming, involving several centrifugations, and since it takes an additional 30 minutes to extract the cells for the pre-RC assay, we deemed the time resolution of the assay too low for these experiments. Therefore, we instead measured the presence of Rum1 and the phosphorylation status of Cdc2 by immunoblotting. Rum1 is a Cdc2 inhibitor which is only expressed in G1 phase (Benito et al., 1998; Correa-Bordes et al., 1997; Moreno and Nurse, 1994) and the Cdc2 kinase is phosphorylated on Tyr15 as DNA replication commences (Hayles and Nurse, 1995; Zarzov et al., 2002). They are therefore good markers of G1 exit and S-phase entry, respectively, and have been used to show UVC-induced delays in our previous studies [fig. 1D in Tvegård et al. and fig. 4A in Nilssen et al. (Tvegård et al., 2007; Nilssen et al., 2003)]. In untreated wild-

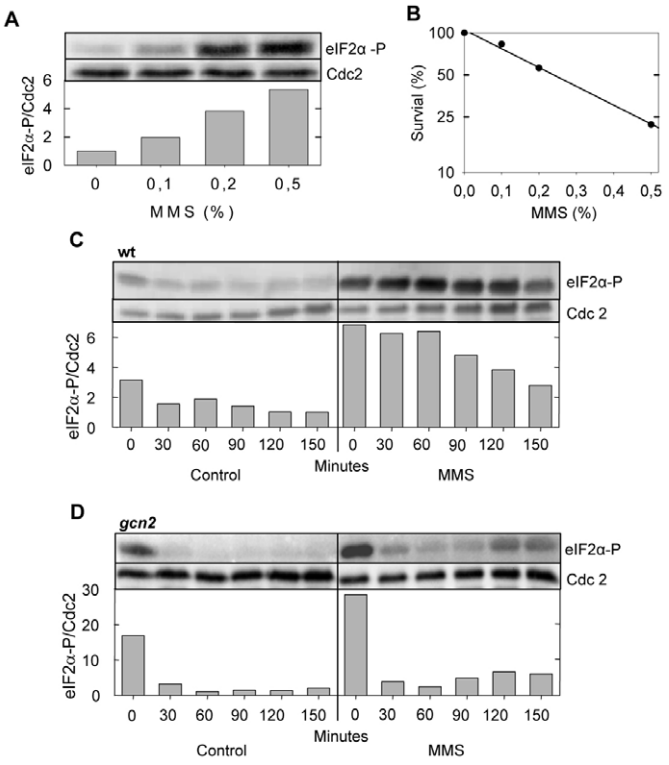

Fig. 3. MMS-induced eIF2α phosphorylation. For details, see Fig. 1. (A) Asynchronous cells exposed to increasing concentrations of MMS. (B) Cell survival after MMS. (C,D) G1-synchronised wild-type (C) and *gcn2* mutant (D) cells were exposed to 0 or 0.15% MMS and incubated for different periods of time after MMS inactivation and removal, as indicated.

type cells Rum1 was present from the start and disappeared around the 60-minutes time point (Fig. 4B), at which time phosphorylated Cdc2 appeared and remained for the duration of the experiment (Fig. 4C). We conclude that the control cells entered S phase at 60 minutes, consistent with previous experiments, including pre-RC assays (above). These observations validate the use of Rum1 and Cdc2 phosphorylation as molecular markers for entry into S phase.

In G1-synchronised wild-type cells treated with MMS Rum1 was still present at 90 minutes (Fig. 4B) and phosphorylated Cdc2 did not appear until 120 minutes (Fig. 4C). Both the continued presence of Rum1 and the delayed phosphorylation of Cdc2 strongly argue that the MMS-treated wild-type cells were delayed in G1, before entry into S phase. In the *gcn2* mutant, both Rum1 degradation (Fig. 4D) and Cdc2 phosphorylation (Fig. 4E) occurred at 60 minutes, both with and without MMS treatment, demonstrating that in the mutant entry into S phase is not delayed by MMS.

We conclude that entry into S phase is delayed in a Gcn2-dependent manner in response to MMS. When the cells have entered S phase they delay by a mechanism independent of Gcn2, most probably the intra-S checkpoint.

Psoralen adducts do not induce phosphorylation of eIF2α or a cell-cycle delay

Psoralen intercalates into double-stranded DNA and creates covalent adducts to the DNA after irradiation with 365 nm UV light (UVA), the so-called PUVA treatment. We exposed G1-synchronised wild-type cells to 10 kJ/m^2 of UVA in the presence of 5 μg/ml psoralen, which resulted in a cell survival of only 1%, whereas the same UVA dose in the absence of psoralen did not appreciably affect survival (data not shown). PUVA treatment did not result in increased phosphorylation of eIF2α (Fig. 5A), even though the low level of cell survival indicates that a significant number of DNA adducts had been introduced.

The mere presence of psoralen (without UVA) blocked G1 cells from progressing into S phase (data not shown) and therefore the cells were washed free of psoralen after UVA exposure to monitor the effect of PUVA treatment on subsequent cell cycle progression. The first sample was collected when the cells were released back into the cell cycle after psoralen removal. PUVA-treated cells were delayed in the cell cycle as determined by flow cytometry (Fig. 5B). The unirradiated control cells increased their DNA content 90 minutes after exposure. This is slightly later than normally observed (Fig. 2D, Fig. 4A) and is most probably due to the psoralen treatment. The broadening of the peak in the DNA histograms of the UVA-exposed cells occurred more slowly than in unexposed cells, suggesting later entry into or through S phase, and they had not completed DNA replication even after 150 minutes. To study this cell-cycle delay in more detail we employed the pre-RC loading assay. The control and irradiated cells followed the same kinetics for pre-RC formation, showing a peak in chromatin-bound Mcm6-GFP at 90 minutes (Fig. 5C). The finding that there was no delay in the pre-RC formation by PUVA strongly suggests that the delay observed by flow cytometry takes place not in G1 phase but exclusively in S phase. This delay is most probably caused by inhibition of replication fork progression by DNA adducts and crosslinks induced by the PUVA treatment. We conclude that PUVA treatment does not lead to eIF2α phosphorylation, nor does it cause a delayed entry into S phase even after inducing so many lesions that the progression of the replication forks is severely inhibited and cell survival is low.

Ionizing radiation does not induce eIF2α phosphorylation or a cell-cycle delay

The exposure of asynchronous cells to increasing doses of ionizing radiation (IR) showed that even a dose of 900 Gy resulted in only a slight increase in the level of eIF2α phosphorylation, when compared with the level of phosphorylation seen after amino acid starvation for 2 hours (Fig. 6A). The cell survival at this dose, about 25% (Fig. 6B), shows that the cells had been significantly damaged. We have earlier shown that cells exposed to IR do not delay cell cycle progression from G1 to S phase (Tvegård et al., 2007). We conclude that IR-induced damage does not induce significant eIF2α phosphorylation, nor does it induce a cell cycle delay in G1.

Discussion

In this work we have investigated how several types of DNA-damaging agents influence the transition from G1 to S phase in fission yeast. We have shown that entry into S phase was delayed in response to oxidative damage by H_2O_2 and alkylation by MMS, but not by IR or PUVA. We have also shown a tight correlation between eIF2α phosphorylation and checkpoint activation, in that the treatments inducing eIF2α phosphorylation also induced a Gcn2-dependent checkpoint – and vice versa. Both the delayed entry into S phase and eIF2α phosphorylation are totally dependent upon Gcn2. These data strengthen the correlation between eIF2α phosphorylation and checkpoint activation but do not prove a causal relationship.

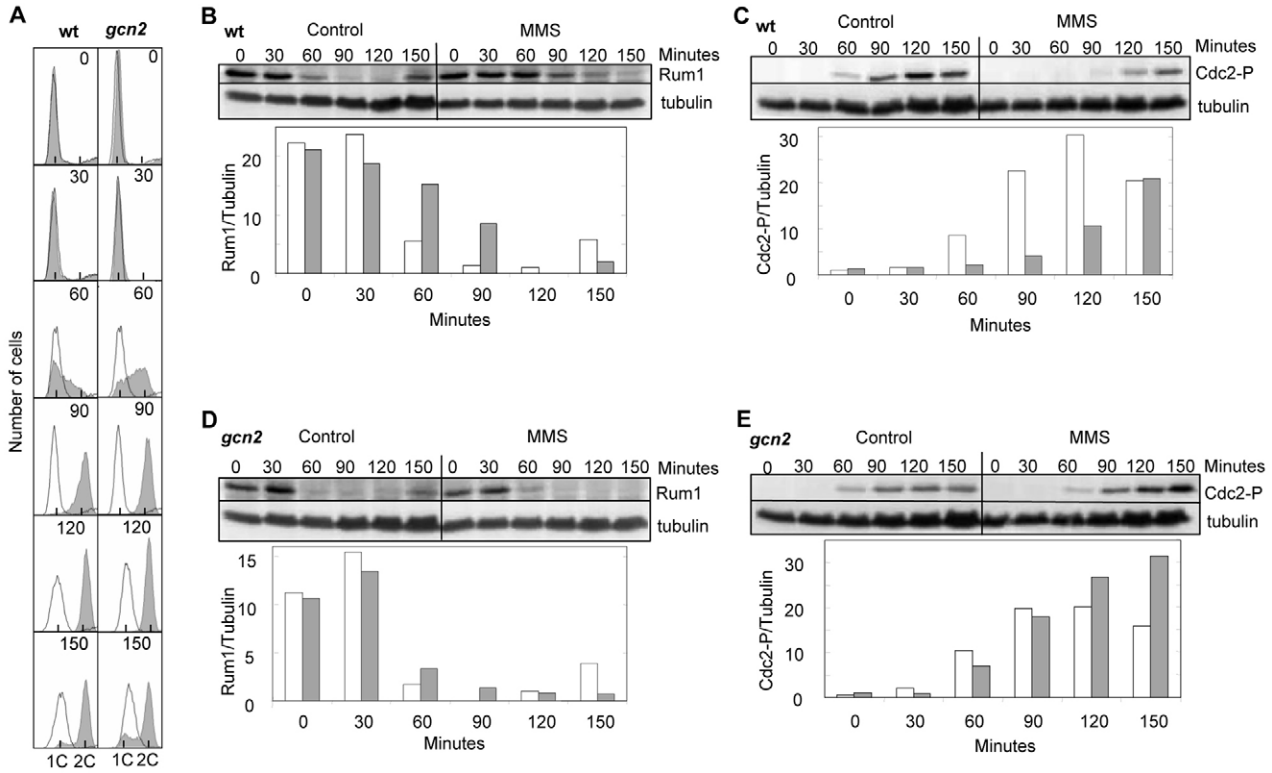

Fig. 4. MMS-induced effects on cell-cycle progression. (A) Flow cytometry histograms of G1-synchronised wild-type (left) and *gcn2* (right) control cells (shaded) and cells treated with 0.15% MMS (unshaded) incubated for the times indicated after MMS treatment. (B-E) Immunoblots (for details, see Fig. 1) of wild-type (B) and *gcn2* (D) cells probed with antibody against Rum1. Immunoblots of wild-type (C) and *gcn2* (E) cells probed with antibody against phosphorylated Cdc2. Antibody against tubulin was used as loading control. Quantifications are shown as white columns for the control and shaded columns for MMS-treated cells.

The importance of eIF2α phosphorylation

eIF2α phosphorylation is an important cellular response to various types of stress in most, if not all, eukaryotic cells. Human cells phosphorylate eIF2α after UVC irradiation, oxidative stress and ER stress (Deng et al., 2002; Jiang and Wek, 2005; Jiang et al., 2003; Wu et al., 2002). Phosphorylation of eIF2α inhibits general translation, allowing the cells to conserve resources and to focus on expression of proteins that are important to cope with the given stress condition. Increasing evidence suggests that inappropriate translation rates of selected proteins in response to stress signals can be deleterious. Changed expression levels of potentially oncogenic proteins or tumour suppressors can result from abnormal translational regulation. In relation to this it is noteworthy that eIF2α is overexpressed in several types of cancer (Clemens, 2004), suggesting that eIF2α is important for cell cycle regulation.

Downregulation of translation would be expected to delay the cell cycle due to a lack of rate-limiting factors required for progression. For example, in several organisms downregulation of G1 cyclins has been observed after different treatments that reduce translation rates (Ghavidel et al., 2007; Grallert et al., 2000; Hamanaka et al., 2005; Polymenis and Schmidt, 1997). However, such a mechanism is an unlikely explanation for the cell cycle delay observed here. The delay in pre-RC formation occurs at a step prior to any requirement for a G1 cyclin and should therefore not be affected by its downregulation. Furthermore, we were able to show that after UVC treatment all known factors required for pre-RC formation are present, but still formation is delayed (Tvegård et al., 2007).

It appears that the duration of the delay of entry into S phase correlates with the duration of an increase in the level of eIF2α phosphorylation. This can best be seen in response to H_2O_2 treatment where the phosphorylation level of eIF2α decreases down to background level at about 120 minutes (Fig. 2B), and at the same time the pre-RC starts to form on the chromatin and the cells move into S phase (Fig. 2E, left panel). This trend can also be seen in response to UVC [Fig. 1D; see also fig. 1C in Tvegård et al. (Tvegård et al., 2007)] and MMS treatment (Fig. 3B, Fig. 4).

The G1-S checkpoint is not induced by DNA damage in general

Gcn2 is clearly not activated by general DNA damage, since we did not observe eIF2α phosphorylation after IR or PUVA. The different treatments that we have employed in this work generate different types of DNA damage. MMS is an alkylating agent that methylates the DNA on different positions, most importantly inducing mutagenic base alterations. H_2O_2 generates highly reactive radicals that can modify the DNA at a wide range of positions. It has been shown that there is a substantial overlap between the genes induced by MMS and by H_2O_2 (Chen et al., 2003) and that these agents may cause cross-protection towards the other. However, the spectrum of genes induced after UVC irradiation in G1 phase, analysed by microarrays, showed no overlap with the genes induced by H_2O_2 (our unpublished data). Thus, there is no obvious connection between gene induction and the checkpoint response after MMS, H_2O_2 and UVC.

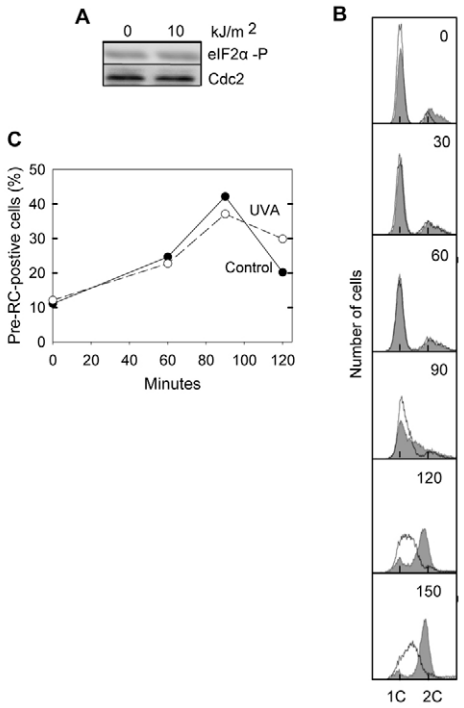

Fig. 5. The effect of PUVA on eIF2α phosphorylation and cell-cycle progression. (A) Immunoblot of G1-synchronised wild-type cells exposed to psoralen and 0 or 10 kJ/m^2 of UVA. (B) Flow cytometry histograms of G1-synchronised wild-type cells irradiated (unshaded) and not irradiated (shaded) and incubated for the times indicated (in minutes) after exposure. (C) Pre-RC assay of G1-synchronised cells at different time points after exposure to 0 (control) or 10 kJ/m^2 of UVA.

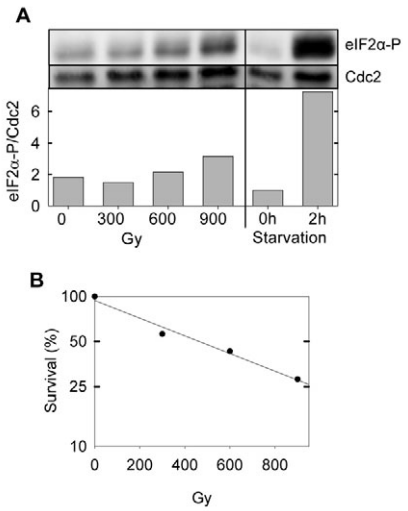

Fig. 6. The effect of ionizing irradiation on eIF2α phosphorylation. (A) Immonoblots of asynchronous cells exposed to increasing doses of IR and a comparison with leucine-starved cells (see Fig. 1). (B) Cell survival after ionizing radiation.

IR induces several types of DNA damage, including single- and double-strand breaks and sugar and base modifications. Psoralen intercalates into double-stranded DNA and forms adducts and inter- and intrastrand DNA cross-links upon exposure to UVA (Cimino et al., 1985). UVC introduces two major types of photodamage to the nucleotide bases: cyclobutane pyrimidine dimers and (6-4) pyrimidone photoproducts.

However, DNA is not the only target of the above-mentioned treatments, since they will also damage other macromolecules in the cells such as RNA, lipids and proteins. PUVA treatment may be the exception, since it does not form adducts in single-stranded RNA (Dall'Acqua et al., 1978). PUVA treatment (this study) and IR (Tvegård et al., 2007) did not affect the phosphorylation level of eIF2α, nor did these treatments delay entry into S phase, as demonstrated by the pre-RC loading assay. The results from these two DNA-damaging treatments allow us to conclude that checkpoint induction and eIF2α phosphorylation do not result from DNA damage in general. This appears to be in contrast to the well described DNA damage checkpoint operating in G2-M, where UVC, MMS, oxidative stress and IR are potent inducers (Cuddihy and O'Connell, 2003). However, we cannot exclude the possibility that a specific form of DNA damage or a repair intermediate that is common to UVC, MMS and H_2O_2 and not to IR and PUVA elicits the G1-S checkpoint response.

In general, little is known about the molecular structures that initiate the signal cascade to activate a checkpoint. However, there is good evidence that single-stranded DNA (ssDNA) covered by a single-strand binding protein (RPA) can serve as such an initiator (Zou and Elledge, 2003). A DNA double-strand break (DSB) is normally processed to give a 3′ overhang of ssDNA which is bound by RPA, and a DSB induces the G2-M checkpoint. The data presented here show that a DSB is not likely to be an inducer of the G1-S checkpoint. Ionizing radiation creates DSBs, yet IR does not induce the G1-S checkpoint. These data also argue that ssDNA is also not inducing the checkpoint, since ssDNA is most probably produced in considerable amounts during the repair of DNA damage after both IR and PUVA.

Our finding that IR does not induce the G1-S checkpoint might be a clue to understanding the nature of checkpoint signalling. In S and G2 phases all types of DNA damage seem to induce a checkpoint activating the kinases Chk1 and/or Cds1 and targeting Cdc2 for phosphorylation, leading to an arrest of entry into mitosis. However, a similar reaction cascade does not occur in G1 phase after the same range of stimuli. Our data reveal a fundamental difference in the handling of DNA damage between G1 and S-G2 phase. What is the molecular characteristic that distinguishes G1 phase from S and G2 when it comes to checkpoint activation?

G1-S checkpoints in other eukaryotes

S. cerevisiae has several G1-S checkpoints induced by agents causing genotoxic stress (Ghavidel et al., 2007; Sidorova and Breeden, 1997; Tercero and Diffley, 2001). In response to MMS a Mec1-Rad53-dependent signalling pathway is induced that phosphorylates, and thereby inhibits, the activity of the SWI6 transcription factor required for the expression of S-phase genes (Sidorova and Breeden, 1997). Another consequence of the activation of Mec1-Rad53 is nuclear accumulation of unspliced tRNA leading to activation of the transcription factor Gcn4, which contributes to execution of the G1 checkpoint through delaying accumulation of the cyclin Cln2 and probably other key regulators of G1 progression (Ghavidel et al., 2007). Whether eIF2α and Gcn2 are involved in this checkpoint is unclear, but eIF2α phosphorylation is known to stimulate translation of Gcn4 (Dever et al., 1992). The G1-S checkpoint described in the present work is clearly different from those mentioned above for budding yeast. However, there is some evidence indicating that a

Gcn2-dependent checkpoint might be active in both yeasts: Phosphorylation of eIF2α correlates with a G1-S delay after MMS treatment both in fission yeast (this work) and in budding yeast (Menacho-Marquez et al., 2007) and eIF2α becomes phosphorylated in response to UVC irradiation both in *S. pombe* (Fig. 1) and in *S. cerevisiae* (data not shown). Furthermore, UVC and other types of stress also induce eIF2α phosphorylation in mammalian cells (Deng et al., 2002) and many of the same treatments are known to result in a G1 delay. Therefore we consider it most likely that the link between Gcn2 activity and cell cycle progression is conserved through evolution. The details of how Gcn2 is activated and how its major known activity, eIF2α phosphorylation, contributes to the cell cycle delay remain to be explored.

Materials and Methods

Yeast strains and cell handling

All the strains are derivatives of the *Schizosaccharomyces pombe* L972 h⁻ strain. The strains used were: *cdc10-M17 h⁻ and leu1-32 h⁺ (P. Nurse); cdc10-M17 gcn2::ura4⁺ ura4-D18 h⁻, cdc10-M17 gcn2::ura4⁺ mcm6-GFP:kanR ade6-M210 ura4-D18 h⁺, cdc10-M17 mcm6-GFP:kanR ade6-M210 ura4-D18 h⁺* (Tvegård et al., 2007); *mcm2-GFP:kanR ade6-M210 ura4-D18 leu1-32 h⁻* (S. Kearsey); *cdc10-M17 mcm2-GFP:kanR ade6-M210 ura4-D18 leu1-32 h⁻* (this study). All strains not carrying the gcn2 deletion will be referred to as wild-type cells. Media and growth conditions were as described previously (Moreno et al., 1991). The cells were grown in liquid Edinburgh minimal medium (EMM), supplemented as required, at 25°C, to a cell concentration of 2–4×10^6/ml (OD_{595} of 0.1-0.2). The *cdc10-M17* cells were synchronised in G1 by incubation at 36°C for 4 hours before return to 25°C. Cell survival was measured by counting colony-forming units on yeast extract (YE) agar plates.

Flow cytometry

The cells were prepared for flow cytometry as described previously (Nilssen et al., 2003) and the samples were analysed with a Becton-Dickinson LSRII.

Pre-RC assay

Extraction of MCM proteins not bound to chromatin was performed as described previously (Gregan et al., 2003; Kearsey et al., 2000).

Starvation

Cells auxotrophic for leucine were grown exponentially in EMM with leucine, washed twice in EMM without leucine and incubated for 2 hours in the same medium.

Irradiation

UVC

UVC (254 nm) irradiation was given at an incident dose rate of about 250 J/m^2/minute in a thin, stirred suspension, as described previously (Nilssen et al., 2003). Synchronised cells were irradiated immediately after release from the Cdc10 block (G1 phase), 60 minutes after release (S phase) or 120 minutes after release (G2 phase).

UVA

Fifteen minutes prior to UVA irradiation 5 μg/ml 8-methoxypsoralen (8-MOP; Sigma) was added to the culture which was kept in the dark. For the synchronised cultures the 8-MOP was present during the last 15 minutes of the 4-hour shift up at 36°C. About 8×10^7 cells were irradiated with 10 kJ/m^2 of UVA (365 nm) at a dose rate of 6 kJ/minute. The irradiation was performed in Petri dishes in a 3 mm thick layer, with vigorous stirring. The cultures were washed twice in EMM, resuspended in EMM and incubated at 25°C before sample collection.

Ionizing radiation

The cells were exposed to 4 MeV electrons from a linear accelerator (Varian Clinac 2100 CD; dose rate ~80 Gy/minute) in Petri dishes in EMM without stirring.

MMS

Exponentially growing asynchronous cells were centrifuged and resuspended at 1×10^8 cells/ml in EMM before methyl methane sulfonate (MMS; Sigma) was added and the culture was incubated for 1 hour at 25°C. For the G1-synchronised cells the MMS was added to a culture of 6×10^6 cells/ml during the last 15 minutes of the shift-up to the restrictive temperature. To inactivate and remove the MMS after exposure, the culture was washed twice in 5% sodium thiosulphate, once in 0.9% sodium chloride and once in EMM before resuspension in EMM and incubation at 25°C before sample collection.

Hydrogen peroxide

H_2O_2 was added during the last 15 minutes of the shift-up to the restrictive temperature. The cells were washed once in EMM to remove the highly reactive H_2O_2 and resuspended in EMM before the samples were collected.

Immunoblots

Total cell extracts were prepared by extraction with TCA as described previously (Caspari et al., 2000), except that β-mercaptoethanol was replaced by dithiothreitol in the sample buffer. Samples were analyzed by SDS-polyacrylamide gel electrophoresis. The proteins were transferred to a PVDF membrane in a semi-dry transfer cell (Bio-Rad) using a transfer buffer containing 0.05 M Tris, 0. 4 M glycine, 20% methanol and 0.1% SDS. The membranes were blocked in 5% membrane blocking agent (Amersham Biosciences) before probing with the relevant antibody. Detection was performed using an ECL kit (Amersham Biosciences) and quantified in a Chemi Genius (Syngene) with the software GeneTools. A relative value of 1 was assigned to the weakest signal in each blot, except where noted. The antibodies used were: anti-PSTAIRE against Cdc2 (Santa Cruz, sc-53) at a dilution of 1:2000; antiserum against phosphorylated eIF2-α (Biosource, no. 44-7282), 1:2000; anti-phospho-cdc2 (Tyr15; Cell Signaling, 9111S), 1:400; anti-Rum1, 1:500 (a kind gift from S. Moreno); anti-α-tubulin (T-5168, Sigma), 1:30,000.

We are grateful to M. O. Haugli and I. Løbersli for technical assistance, to T. Furre for assistance in operating the linear accelerator and to S. Moreno for Rum1 antiserum. This work was supported by the Norwegian Cancer Society, The Research Council of Norway and by the South-Eastern Norway Regional Health Authority.

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