p56chk1 protein kinase is required for the DNA replication checkpoint at 37°C in fission yeast

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Fission yeast p56chk1 kinase is known to be involved in the DNA damage checkpoint but not to be required for cell cycle arrest following exposure to the DNA replication inhibitor hydroxyurea (HU). For this reason, p56chk1 is considered not to be necessary for the DNA replication checkpoint which acts through the inhibitory phosphorylation of p34\(^{\text{cdc2}}\) kinase activity. In a search for Schizosaccharomyces pombe mutants that abolish the S phase cell cycle arrest of a thermosensitive DNA polymerase \(\delta\) strain at 37°C, we isolated two chk1 alleles. These alleles are proficient for the DNA damage checkpoint, but induce mitotic catastrophe in several S phase thermosensitive mutants. We show that the mitotic catastrophe correlates with a decreased level of tyrosine phosphorylation of p34\(^{\text{cdc2}}\). In addition, we found that the deletion of chk1 and the chk1 alleles abolish the cell cycle arrest and induce mitotic catastrophe in cells exposed to HU, if the cells are grown at 37°C. These findings suggest that chk1 is important for the maintenance of the DNA replication checkpoint in S phase thermosensitive mutants and that the p56chk1 kinase must possess a novel function that prevents premature activation of p34\(^{\text{cdc2}}\) kinase under conditions of impaired DNA replication at 37°C.

Keywords: cell cycle/chk1/DNA replication/heat shock/ S.pombe

Introduction

The integrity of the genome is ensured by accurate DNA replication and repair and correct ordering of cell cycle events by control pathways known as checkpoints (Hartwell and Weinert, 1989). Studies in fission yeast have provided a great deal of insight into the mechanism that couples mitosis to completion of DNA replication and DNA repair in eukaryotes (Nurse, 1994).

The temporal order of S phase and mitosis is dependent on the state of the p34\(^{\text{cdc2}}\)-cyclin B complex whose active form brings about mitosis (Hayles et al., 1994). The complex is rendered inactive after Start by the wee1 and mik1 kinases which phosphorylate p34\(^{\text{cdc2}}\) on tyrosine 15 (Y15) (Gould and Nurse, 1989; Lundgren et al., 1991; Smythe and Newport, 1992; Hayles and Nurse, 1995). Subsequent dephosphorylation by the cdc25 phosphatase activates the p34\(^{\text{cdc2}}\)-cyclin B complex allowing passage into mitosis (Moreno et al., 1989). Thus, in cycling cells, the timing of mitosis is determined by a gradual change in the wee1/cdc25 ratio. This ratio influences the cell size at division and maintains the dependence of mitosis on the completion of DNA replication (Enoch and Nurse, 1990; Moreno et al., 1990). Recently, it has been shown that during the S phase cell cycle block at the restrictive temperature of the thermosensitive cdc22-M45 mutant, the cdc25 phosphatase accumulates to high levels although its basal activity remains low. This indicates that the DNA replication checkpoint operates despite the accumulation of cdc25 protein (Kovelman and Russell, 1996).

Fission yeast mutants deficient in the DNA replication checkpoint have been isolated which fail to arrest the cell cycle in the presence of the DNA replication inhibitor hydroxyurea (HU) and thereby undergo mitotic catastrophe (hus mutants). Analysis of hus mutants provided evidence for a partial overlap between DNA replication and DNA damage checkpoints since many of these mutants were also affected in the ability to arrest the cell cycle in G2 phase after DNA damage, resulting in the radiation-sensitive phenotype (rad mutants). However, in fission yeast, the radiation checkpoint seems not to involve the Y15 phosphorylation of p34\(^{\text{cdc2}}\). Indeed, mutations affecting elements involved in Y15 regulation do not abolish the G2 delay when cells are irradiated (Al-Khodairy and Carr, 1992; Enoch et al., 1992; Barbet and Carr, 1993; Sheldrick and Carr, 1993; Al-Khodairy et al., 1994).

The fission yeast checkpoint gene chk1\(^{+/+}\)/rad27\(^{+/+}\) has been proposed to be solely involved in the DNA damage checkpoint, as deletion of this gene abolishes cell cycle arrest after irradiation (rad phenotype) but does not abolish cell cycle arrest when cells are exposed to the DNA replication inhibitor HU at 30°C (absence of hus phenotype) (Walworth et al., 1993; Al-Khodairy et al., 1994; Carr, 1995). Recently, it has been shown that p56chk1 is phosphorylated when cells are treated with different DNA-damaging agents (Walworth and Bernards, 1996). We reported that deletion of the chk1 gene in S phase thermosensitive mutants abolishes cell cycle arrest and leads to mitosis in the absence of complete DNA replication under non-permissive conditions (Francesconi et al., 1995). These findings were intriguing since deletion of chk1 does not result in the hus phenotype when the cells are treated with HU under normal growth conditions (30°C).

Here we show that the response to HU treatment is dependent on the growth temperature, as cells deleted for the chk1 gene undergo mitotic catastrophe if exposed to HU at 37°C. Furthermore, we isolated chk1 mutant alleles that do not abolish cell cycle arrest after UV irradiation and that are not sensitive to the alkylating agent methylmethane sulfonate (MMS). These alleles induce mitotic catastrophe either in the presence of HU at 37°C or when combined with S phase thermosensitive mutants. Thus, we demonstrate that chk1 protein kinase has a function in the DNA replication checkpoint.
replication checkpoint that is unrelated to its function in the DNA damage checkpoint. The role of chk1 in the DNA replication checkpoint is apparent at 37°C, suggesting a possible link between this checkpoint and the heat-shock response.

Results

Genetic screening for mutants inducing lethal mitosis in a DNA polymerase δ thermostable strain

We performed a screening in order to identify mutations that abolish the S phase cell cycle arrest of strain \( \text{pol} \delta \text{ts}3 \) at the non-permissive temperature. This strain carries a thermostable mutation in the gene encoding DNA polymerase δ (\( \text{pol} \delta 3^+ \)) and, when shifted to 37°C, cells arrest in S phase with a cdc (cell division cycle) terminal phenotype (Pignède et al., 1991; Francesconi et al., 1993). A total of 16 000 clones derived from the mutagenized \( \text{pol} \delta \text{ts}3 \) strain were grown at 37°C and scored under the microscope for the absence of cdc terminal phenotype (Figure 1A). Nine clones were isolated which lacked the cdc terminal phenotype but were still thermostable for growth. We first analysed these clones for genetic linkage to some of the known checkpoint genes. In order to perform this analysis, we crossed the \( \text{pol} \delta \text{ts}3 \) thermosensitive strain with the following checkpoint mutant strains: \( \text{cdc}2-3 \), \( \text{hus}1-14 \), \( \text{rad}1-1 \) and \( \text{dchk}1 \) (Table I) (Enoch and Nurse, 1990; Al-Khodairy and Carr, 1992; Enoch et al., 1992; Walworth et al., 1993; Al-Khodairy et al., 1994). When possible, double mutants were isolated and their phenotypes analysed at the restrictive temperature (Table II). We found that the \( \text{pol} \delta \text{ts}3 \) allele is synthetic lethal (SL) at the permissive temperature with both \( \text{hus}1-14 \) and \( \text{rad}1-1 \) alleles (Table II). The double mutants that we could isolate (\( \text{pol} \delta \text{ts}3 \text{cdc}2-3 \) and \( \text{pol} \delta \text{ts}3 \text{dchk}1 \)) were then crossed with the nine clones derived from the screening in order to determine genetic linkage. We found that two out of nine clones from the screening which showed the cut (cell untimely torn) phenotype at 37°C (Figure 1B) were harbouring the \( \text{pol} \delta \text{ts}3 \) allele and a mutation in the \( \text{chk}1 \) gene (we will refer to the \( \text{chk}1 \) mutations as \( \text{chk}1-1 \) and \( \text{chk}1-2 \) alleles). Furthermore, none of the nine clones regained the cdc terminal phenotype at 37°C when transformed with a plasmid carrying the wild-type checkpoint gene \text{cut}5^+ (Saka and Yanagida, 1993; Saka et al., 1994).

To extend the analysis of the genetic interaction between the different checkpoint mutants and S phase \( \text{cdc} \) mutants, we crossed the thermostable strains \( \text{pol} \delta \text{ts}1, \text{pol} \delta \text{ts}2, \text{pol} \delta \text{ts}1 \) (DNA polymerase \( \alpha \text{cdc} \) mutant, our unpublished result), \( \text{cde}17 \) (DNA ligase), \( \text{cde}20 \) and \( \text{cde}24 \) with the checkpoint mutant strains listed in the previous paragraph (Nasmyth and Nurse, 1981; Damagnez et al., 1990; Francesconi et al., 1993). The phenotype of double mutants was then analysed and results are summarized in Table II. Synthetic lethality at the permissive temperature of 25°C was observed only in the combination of \( \text{pol} \delta \) and \( \text{pol} \alpha \) mutants with either \( \text{rad}1-1 \) or \( \text{hus}1-14 \) checkpoint mutations. All the others combinations were viable at 25°C and showed the cut phenotype at 37°C (Table II).

Primary characterization of \( \text{pol} \delta \text{ts}3 \text{chk}1-1 \) and \( \text{pol} \delta \text{ts}3 \text{chk}1-2 \) double mutants

The double mutants \( \text{pol} \delta \text{ts}3 \text{chk}1-1 \) and \( \text{pol} \delta \text{ts}3 \text{chk}1-2 \) isolated from the screening described above were transformed with a plasmid carrying the wild-type \( \text{chk}1^+ \) gene. Transformants regained the cdc terminal phenotype at 37°C. Double mutants were then back-crossed to the \( \text{pol} \delta \text{ts}3 \) strain and finally crossed with wild-type, allowing isolation of \( \text{chk}1-1 \) and \( \text{chk}1-2 \) single mutants. Combination of either of the two \( \text{chk}1 \) alleles with the \( \text{pol} \delta \text{ts}3 \),
Despite incomplete DNA replication (Figure 2C and D), shown. We then investigated whether these mutants were able to arrest the cell cycle after UV irradiation at the leading to accumulation of cells with the cut phenotype at 37°C (not shown) (Nasmyth et al., 1981; Francesconi et al., 1993, 1995). We further analysed the phenotype of pol8ts2 mutant alleles or with other known S phase mutants (polats1, cdc17, cdc20 and cdc24) results in cells with the cut phenotype at 37°C (not shown) (Nasmyth and Nurse, 1981; Francesconi et al., 1993, 1995).

We further analysed the phenotype of pol8ts3 chk1 double mutants at 37°C in synchronized cultures. Unlike strain pol8ts3 (Figure 2B), synchronizations by centrifugal elutriation of double mutant strains pol8ts3 chk1-1 and pol8ts3 chk1-2 at 37°C showed that the cells enter mitosis despite incomplete DNA replication (Figure 2C and D), leading to accumulation of cells with the cut phenotype (not shown). This behaviour is similar to that observed for the pol8ts3 chk1::ura4+ double mutant (Francesconi et al., 1995). Thus, we conclude that in double mutant strains at the non-permissive temperature, mitosis is uncoupled from DNA replication.

We then obtained and sequenced chk1 alleles from both mutants by PCR amplification. Allele chk1-1 has a single point mutation resulting in the substitution of Glu92 by aspartic acid. This amino acid is conserved in the catalytic domain of many serine/threonine protein kinases, suggesting that this mutant could be altered in the kinase activity. Allele chk1-2 also has a single point mutation changing Ile484 to threonine. This mutation, at the very carboxy-terminus of the protein, is not located in the catalytic domain of protein kinases.

### Table I. Strains used in this study

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<th>Source</th>
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<td>a</td>
</tr>
<tr>
<td>dchk1 (SP1182)</td>
<td>hþ chk1::ura4- ura4-D18</td>
<td>b</td>
</tr>
<tr>
<td>chk1-1</td>
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<td>c</td>
</tr>
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Table II. Relevant phenotype of double mutants

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<th>rad1-1</th>
<th>dchk1</th>
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<td>cut 37°C</td>
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</tr>
<tr>
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<td>cut 37°C</td>
<td>cut 37°C</td>
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</tr>
<tr>
<td>cdc24-M38</td>
<td>cut 37°C</td>
<td>cut 37°C</td>
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<td>cut 37°C</td>
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Characterization of the DNA damage checkpoint in chk1-1 and chk1-2 mutants

We analysed the radiation sensitivity of chk1-1 and chk1-2 single mutants at different temperatures. Surprisingly, we found that, unlike the dchk1 strain (chk1 deletion), chk1-1 and chk1-2 mutants are not radiation sensitive (data not shown). We then investigated whether these mutants were able to arrest the cell cycle after UV irradiation at the same temperature at which they had been isolated, 37°C. Like the wild-type strain, strains chk1-1 and chk1-2 show a decrease in septation index after irradiation with 200 J/m² at 37°C, indicating that the DNA repair checkpoint is intact (Figure 3A). In contrast, control strain dchk1 had completely lost the DNA damage checkpoint as previously shown (Walworth et al., 1993; Al-Khodairy et al., 1994). We further analysed the radiation checkpoint in the synchronized double mutant pol8ts3 chk1-1 at 37°C. Similarly to the wild-type strain, the double mutant delays mitosis after irradiation in a dose-dependent manner, confirming that the radiation checkpoint is active despite mutations in pol8 and chk1 genes (Figure 3B). The same
Fig. 2. Synchronized polδts3 chk1-1 and polδts3 chk1-2 double mutants do not arrest the cell cycle at 37°C despite incomplete DNA replication. (A) Synchronized polδts3 at 25°C; (C) % septated cells; (B) synchronized polδts3 at 37°C; (D) % septated cells, (D) % surviving cells; (C) synchronized polδts3 chk1-1 at 37°C, (E) % septated cells, (F) % surviving cells; (D) synchronized polδts3 chk1-2 at 37°C, (G) % septated cells, (H) % surviving cells. Synchronized double mutants at the permissive temperature of 25°C behave as a polδts3 single mutant (A).

Fig. 3. Mutant alleles chk1-1 and chk1-2 are proficient for the DNA damage checkpoint after UV irradiation at 37°C (the same results were obtained at 30°C). (A) Asynchronous cultures of chk1-1 and chk1-2 single mutants arrest the cell cycle after UV irradiation at 200 J/m² similarly to wild-type (wt) (irradiation at 100 J/m² gave similar results). (B) Synchronous culture of the polδts3 chk1-1 double mutant (lower panel) delays mitosis after irradiation at 37°C in a dose-dependent manner similarly to wild-type strain (upper panel) (the same results were obtained for the polδts3 chk1-2 double mutant).
results were obtained for the pol3 ts3 chk1-2 double mutant (not shown). Furthermore, chk1-1 and chk1-2 mutants are not sensitive to the alkylating agent MMS which induces DNA strand breaks. Mutant strains behave similarly to the wild-type when exposed to MMS at 37°C (a behaviour similar to the wild-type strain was also observed at 30°C). In contrast, the control strain dchk1 is highly sensitive to exposure to MMS (Figure 4A). Unlike the dchk1 strain, the partial sensitivity to MMS of the wild-type and chk1 alleles does not result from illegitimate passage into mitosis as shown by 4',6'-diamidino-2-phenylindole (DAPI) staining of the cells after 4 h of MMS treatment at 37°C (Figure 4B). These experiments clearly demonstrate that chk1-1 and chk1-2 alleles are proficient for the DNA repair checkpoint.

**HU sensitivity of chk1 mutants**

Our results, suggesting a role for p56^chk1^ kinase in the DNA replication checkpoint, are in conflict with previous reports that the chk1-deleted strain is insensitive to the DNA replication inhibitor HU (Walworth *et al.*, 1993; Al-Khodairy *et al.*, 1994). We re-examined this phenotype at 30 and 37°C. Cells were grown in YEA medium and 12 mM HU was added at time 0 and after 3 h of incubation at 30 and 37°C. The dchk1 and chk1-1 mutants behaved similarly to the wild-type strain in the presence of HU at 30°C, as demonstrated by survival, septation index and cut index of the different strains (Figure 5A, panels a, b and c). The control strain hus1-14 rapidly lost viability at both 30 and 37°C and cells showed the cut phenotype as previously described (Enoch *et al.*, 1992). The dchk1 strain is slightly more sensitive to exposure to 12 mM HU than are wild-type cells at 30°C. This sensitivity does not result from the absence of the DNA replication checkpoint as shown by the septation and cut index and in agreement with a previous report (Figure 5A) (Al-Khodairy *et al.*, 1994). In addition, the dchk1 strain showed a slight increase of cells with the cut phenotype after 10 h of incubation when compared with wild-type and chk1-1 strains (Figure 5A, panel c). Since FACS analysis showed that DNA replication was completed at 30°C after 9 h of incubation in the presence of HU (not shown), the percentage of dchk1 cells with the cut phenotype after 10 h of incubation could depend on the lack of the G2 DNA repair checkpoint. In marked contrast, dchk1 and chk1-1 mutants lost viability when exposed to 12 mM HU at 37°C (Figure 5B, panel a). Mutant cells showed first an attempt to block the cell cycle, as shown by the decrease in septation index during the first 3 h of treatment, followed by an abnormal increase in the percentage of septated cells (Figure 5B, panel b). DAPI stainings of mutant cells exposed to HU at 37°C clearly showed an accumulation of cells with the cut phenotype (Figure 5B, panel c and e). The septation and cut index of chk1 mutants increased after 4 h of incubation while the wild-type strain started to septate after 6 h of incubation in the same experimental conditions and did not accumulate cells with the cut phenotype (Figure 5B, panels b and c). FACS analysis showed that after 4 h of incubation at 37°C, cells exit the G1 block imposed by HU and enter S phase (Figure 5B, panel d). In the wild-type strain, the S phase in the presence of HU took place in ~3 h and it is during this time period that chk1 mutants start to lose

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**Fig. 4.** Mutant strains chk1-1 and chk1-2 are not sensitive to MMS. (A) Survival after exposure to 0.01% MMS at 37°C for different times. (B) Nuclear morphology of cells treated with 0.01% MMS at 37°C for 4 h; arrows indicate aberrant mitosis; bar represents 10 μm.
Fig. 5. HU sensitivity of chk1 mutants. (A) chk1 mutants are not sensitive to transient exposure to HU at 30°C: survival (a), septation index (b) and cut index (c) of wild-type, hus1-14, dchk1 and chk1-1 strains. (B) chk1 mutants are sensitive to transient exposure to HU at 37°C: survival (a), septation index (b) and cut index (c) of wild-type, hus1-14, dchk1 and chk1-1 strains. (d) FACS analysis performed on wild-type, dchk1 and chk1-1 cells at 0, 4, 5, 6 and 7 h of incubation at 37°C in the presence of HU. (e) Percentage of the different cut phenotypes observed in dchk1 and chk1-1 mutants at 37°C in the presence of HU.
viability and to show the cut phenotype. At 6 h, cells are clearly in S phase and both chk1 mutants have ~25% of septated cells and 15% of cells with the cut phenotype. It is worthwhile underlining that the chk1-1 mutant, which is proficient for the DNA repair checkpoint (Figures 3 and 4), is sensitive to HU at 37°C similarly to the chk1-deleted strain, suggesting that the observed phenotypes do not depend on the absence of a functional DNA damage checkpoint. In addition, the dchk1 strain, which lacks the DNA repair checkpoint at both 30 and 37°C, does not show sensitivity to HU at 30°C.

These experiments demonstrate that, unlike the has1-14 mutant that is sensitive to the block in late G1/early S phase at all the temperatures, the chk1 mutants are sensitive to HU in middle-late S phase at 37°C. This result is consistent with the observation that the chk1 checkpoint is essential to maintain the S phase cell cycle block of DNA polymerase δ thermosensitive strains whose DNA replication begins but is not completed, leading to cells arrested with a DNA content comprised between 1C and 2C (Francesconi et al., 1993, 1995). Thus, we can conclude that the chk1 checkpoint is essential for coupling mitosis to completion of S phase when DNA replication is impaired at 37°C. Furthermore, since at 37°C in the presence of HU there was an attempt to stop the cell cycle, the chk1 checkpoint might act after inhibitory tyrosyl phosphorylation of p34cdc2 has occurred in order to prevent premature activation of the kinase when DNA replication is impaired at 37°C.

**Analysis of p34cdc2 tyrosine phosphorylation in polΔts3 and polΔts3 chk1 mutants**

We analysed the level of tyrosine phosphorylation of p34cdc2 in strains polΔts3 and polΔts3 chk1-1. Cells in log phase were shifted to 37°C and protein extracts were prepared at 1 h intervals after the shift. An equal amount of total protein was incubated with p9ckKS beads (Zhang et al., 1995) that precipitate p34cdc2 kinase. Precipitated p34cdc2 was probed with anti-phosphotyrosine antibodies and, after stripping, re-probed with anti-cdc2 PSTAIRE antibodies (Figure 6A). Western blot shows that in the polΔts3 single mutant, which arrests the cell cycle at the non-permissive temperature, the level of tyrosine-phosphorylated p34cdc2 increased during the shift (Figure 6A, a and b). This result is in agreement with the results obtained for cdc20 and cdc22 S phase thermosensitive mutants (Hayles and Nurse, 1995). On the contrary, the double mutant strain, which at 37°C does not display cell cycle arrest, shows a level of phosphorylated p34cdc2 which initially increased after 6 h of shift and then decreased considerably (Figure 6A, c and d). The fluctuation of the amount of tyrosine-phosphorylated p34cdc2 in the double mutant strain suggests that, as previously discussed for the HU experiment, chk1 acts after the DNA replication checkpoint has been activated and that it is necessary to maintain the inhibitory Y15 phosphorylation of p34cdc2–cyclin B complex during the DNA replication block at 37°C.

We further investigated the level of phosphotyrosine by analysing the phosphoamino acid content of p34cdc2 precipitated from an equal amount of polΔts3 and polΔts3 dchk1 mutant cells shifted to 37°C. We chose the polΔts3 dchk1 double mutant strain because cells at 37°C enter mitosis more quickly when compared with the polΔts3 chk1-1 double mutant, probably because of the different nature of the chk1 mutation (deletion of the gene versus point mutation). The phosphoamino acid detection and quantification showed that the phosphotyrosine content of p34cdc2 precipitated from the polΔts3 mutant and revealed with anti-cdc2 PSTAIRE antibodies. (b) p34cdc2 precipitated from the polΔts3 mutant and revealed with anti-phosphotyrosine antibodies. (c) p34cdc2 precipitated from the polΔts3 chk1-1 double mutant and revealed with anti-cdc2 PSTAIRE antibodies. (d) p34cdc2 precipitated from the polΔts3 chk1-1 double mutant and revealed with anti-phosphotyrosine antibodies. (B) Phosphoamino acid analysis of in vivo labelled p34cdc2 precipitated from polΔts3 and polΔts3 dchk1 strains shifted to 37°C for 6 h. The control cdc25 strain was shifted to 37°C for 4 h. Y, phosphotyrosine; T, phosphothreonine; S, phosphoserine. TLC (thin layer cellulose) plates were exposed to Molecular Dynamics Screen. Screens were scanned using a Molecular Dynamics Phosphomager. Quantification was done with ImageQuant Software.

**Discussion**

The chk1+/rad27+ gene was identified previously by two different screenings: the first screening was designed in
order to look for multicopy suppressors of the cold-sensitive allele cdc2-4r4 (Walworth et al., 1993), and the second screening was aimed at finding mutants that were sensitive both to radiation and to a transient temperature shift in a cdc17-α2 background (ts DNA ligase) (Al-Khodairy et al., 1994). It was considered that chk1+/rad27+ is necessary for the DNA damage checkpoint but is not required for the DNA replication checkpoint. We previously showed that chk1 deletion induces rapid death and passage into mitosis despite incomplete DNA replication in several S phase thermosensitive mutants (Francesconi et al., 1995). Similarly, combination of the cdc2-3w mutation, which abolishes the checkpoint for DNA replication but not that for DNA repair, with S phase cdc mutants results in mitotic catastrophe under non-permissive conditions (Table I). The chk1 requirement for the cell cycle block of S phase cdc mutants was difficult to reconcile with the observation that chk1-deleted cells are still able to block the cell cycle in the presence of the DNA replication inhibitor HU (Al-Khodairy et al., 1994).

Here we report the identification of two chk1/rad27 alleles that induce mitotic catastrophe in the DNA polymerase δ thermosensitive strain and in several S phase cdc mutants. We demonstrate that the identified chk1 alleles are proficient for the DNA damage checkpoint, implying that the mitotic catastrophe observed in the S phase cdc mutant background does not result from loss of this checkpoint. Thus, these chk1 alleles reveal a new function for the p56chkl protein kinase in the DNA replication checkpoint. We re-examined the behaviour of a chk1-deleted strain and of the identified chk1-1 mutant in the presence of HU. We found that at 37°C mutant cells are unable to maintain the cell cycle block imposed by HU treatment, suggesting that chk1 kinase is not required for activating the DNA replication checkpoint but for keeping it active when DNA replication is impaired at 37°C. Thus, the chk1+ gene product would appear to be a necessary element of the DNA replication checkpoint under heat shock conditions. We previously suggested that in the pol1δts3 chk1::ura4+ double mutant both the DNA replication and the repair checkpoints were inactive, leading to uncontrolled entry into mitosis at 37°C. This interpretation implied that DNA polymerase δ is required for the replication feedback control (Francesconi et al., 1995). In the light of the results presented here, it is clear that the absence of the replication checkpoint in double mutants at 37°C is due to mutations in the chk1 gene rather than in pol1. However, we report (Table I) that hus1-14 and rad1-1 checkpoint mutants, which are deficient in both DNA replication and repair checkpoints, are synthetic lethal at permissive temperature with either DNA polymerase α or DNA polymerase δ thermosensitive cdc mutants. The synthetic lethality is also not observed with the cdc2-3w mutant, which is only deficient in the replication checkpoint, nor with the chk1 null allele mutant, which is affected at 25°C only in the repair checkpoint. Altogether these results suggest that the pol1 and pol1δ mutants have a defect in DNA replication at the permissive temperature requiring an additional function of hus1 and rad1 essential to allow cell survival. Indeed, an additional function necessary for recovery from S phase arrest has been proposed for the hus1+ and rad1+ genes since the mutants lose viability dramatically in the presence of HU even before cells undergo mitotic catastrophe (Al-Khodairy and Carr, 1992; Enoch et al., 1992; Rowley et al., 1992; Al-Khodairy et al., 1994). The observation that the recovery function of hus1 and rad1 could be essential in DNA polymerase α and δ mutants at the permissive temperature suggests that these replicative enzymes are important targets for monitoring a correct progression through S phase.

The chk1 alleles described in this report are proficient for the DNA damage checkpoint, but induce mitotic catastrophe in a cdc17 background (ts DNA ligase). This suggests that monitoring unligated DNA does not require the DNA damage checkpoint. This observation is in agreement with the recent report of mutational analysis of checkpoint rad1+ (Kanter-Smoler et al., 1995). Mutant rad1-S3 retains the DNA damage checkpoint but not the DNA replication checkpoint and induces a phenotype of rapid death and lethal passage into mitosis in the cdc17 background. Interestingly, it has been reported that p56chkl kinase is constitutively phosphorylated in a cdc17 strain at the permissive temperature of 25°C (Walworth and Bernards, 1996).

We found p56chkl to be required for the cell cycle arrest in middle/late S phase at 37°C. This indicates that chk1 protein kinase couples cell cycle control to the heat shock response and suggests that functions and interactions of checkpoint proteins in either the DNA repair or DNA replication checkpoint pathways are modulated in response to different growth conditions. It is known that in S.pombe the heat shock response and cell cycle control are connected (Polanshek, 1977). The fission yeast homologue of Hsp90, a member of a family of proteins involved in stress responses, has been found to bind to and to be required for wee1 kinase activity at all temperatures (Algire et al., 1994). More recently, a heat shock-inducible cyclophilin-like protein was found to suppress the cell cycle defect of the wee1-50 cdc25-22 win1-1 triple mutant (Weisman et al., 1996).

We found that the level of the tyrosine-phosphorylated inactive form of p34cdc2 is reduced in pol1δts3 chk1 double mutants at 37°C when compared with the pol1δts3 single mutant, suggesting that the p56chkl protein kinase could act in the DNA replication checkpoint under conditions of temperature stress by modulating the activities of the wee1 and/or cdc25 p34cdc2 regulators. Alternatively, p56chkl could modify the p34cdc2–cyclin B complex, allowing maintenance of its phosphorylated inactive form at 37°C.

Materials and methods

Yeast methods and media

The S.pombe strains used in this study are listed in Table I. Standard genetic procedures and media were as described (Gutz et al., 1974; Moreno et al., 1991). We refer to YEA medium, in which cells have been grown, as the conditioned medium.

Mutagenesis was performed by collecting 1×10^8 mid-log phase cells grown in YEA at 25°C and resuspending them in 1 ml of 0.2 M NaPO₃, pH 7, 2.5% EMS (methane-sulfonic acid ethyl ester. Sigma). At this EMS concentration cell survival was 50%. The cell suspension was incubated for 1 h at room temperature, 5 ml of 6% Na-thiosulfate were added and cells were washed five times in YEA before plating. Plates were incubated at 25°C until colony formation. A total of 16 000 clones were replicated on YEA plates and incubated at 37°C overnight.

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Replicated clones were scored under the microscope in a search for those which did not display the cdc terminal phenotype.

Preparation of synchronous cultures was performed by centrifugal elutriation as previously described (Francesconi et al., 1995). Synchronization was followed every 20 min from time 0 by estimation of the percentage of cells under a white light phase contrast microscope. Every hour from time 0 cells from the cultures at 37°C were collected, diluted and plated on YEA at 25°C in order to quantify cell survival.

Measurements of the radiation checkpoint in asynchronous cultures were performed by growing cells in YEA at 37°C to 2 × 10⁶ cells/ml; cells were collected, resuspended in water at 5 × 10⁵ cells/ml and split into two samples one of which was irradiated with 200 J/m² in a Stratagene Stratalinker UV source while the other sample served as a non-irradiated control. Cells were then re-inoculated in conditioned medium at 37°C at 2 × 10⁵ cells/ml and percentages of septated cells were followed every 30 min.

Measurements of the radiation checkpoint in synchronized cultures were performed by selecting early G2 populations from cultures grown in YEA to 1 × 10⁷ cells/ml at 25°C. Early G2 cells were resuspended in water at 5 × 10⁵ cells/ml and each sample was split into three aliquots, two of which were irradiated with 50 and 100 J/m², respectively, while the third one served as a non-irradiated control. Non-irradiated and irradiated cells were re-inoculated in conditioned medium at 2 × 10⁶ cells/ml at 37°C; the septation index was followed every 20 min under a white light phase contrast microscope.

Determination of (Fluka) sensitivity was performed by growing cells in YEA to mid-log phase at 37°C; at time 0, MMS was added to 0.01% final concentration; from time 0 cell samples were collected every hour, MMS was inactivated with 5% Na thiosulfate, cells were diluted and plated on YEA at 37°C in order to quantify cell survival.

For HU (Sigma) experiments, cultures were grown to 2 × 10⁶ cells/ml and then split into two samples, one of which was re-incubated at 30°C while the other was shifted to 37°C. After 1 h of incubation, HU was added to 12 mM final concentration (time 0) and again it was added after 3 h (time 3). From time 0, cell samples were collected every hour, diluted and plated for survival estimation. The septation index was followed under a white light phase contrast microscope. An aliquot of each cell sample was treated for DAPI staining in order to determine the percentage of cells with the cut phenotype, and an aliquot was treated to estimate the cellular DNA content as previously described using a Becton-Dickinson FACScan (Costello et al., 1986).

Protein manipulation

Native total protein extracts from S.pombe were prepared as previously described (Moreno et al., 1991). One mg of protein extract was incubated with 25 μl of p95 kinase Sepharose beads at 4°C for 2 h, washed four times with 1 ml of HB buffer and finally resuspended in 30 μl of HB buffer plus an equal volume of 2× sample buffer. After boiling, samples were electrophoresed on an SDS-polyacrylamide gel (Laemmli, 1970). Western blotting was carried out using Immobilon-P (Millipore). Membranes were probed with anti-phosphotyrosine antibodies PY20 (Transduction Laboratories) and, after stripping, with anti-cdc2 (PSTAIRE) antibodies (Santa Cruz Biotechnology). Immunoblots were revealed by ECL (Amersham). Ortho [32P]Phosphate labelling of cells was performed as previously described (Moreno et al., 1991) using 2.5 mCi of radioactivity. Labelled p34cdc2 was precipitated with p95 kinase Sepharose beads as described above, electrophoresed on an SDS–polyacrylamide gel and transferred onto Immobilon-P (Millipore). The radioactive band was confirmed to be the p34cdc2 protein by probing with anti-cdc2 (PSTAIRE) antibodies and was used for the two-dimensional phosphoamino acid analysis as previously described (Cooper et al., 1983).

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