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A role for DNA primase in coupling DNA replication to DNA damage response

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The temperature-sensitive yeast DNA primase mutant *pri1-M4* fails to execute an early step of DNA replication and exhibits a dominant, allele-specific sensitivity to DNA-damaging agents. *pri1-M4* is defective in slowing down the rate of S phase progression and partially delaying the G_1–S transition in response to DNA damage. Conversely, the G_2 DNA damage response and the S–M checkpoint coupling completion of DNA replication to mitosis are unaffected. The signal transduction pathway leading to Rad53p phosphorylation induced by DNA damage is proficient in *pri1-M4*, and cell cycle delay caused by Rad53p overexpression is counteracted by the *pri1-M4* mutation. Altogether, our results suggest that DNA primase plays an essential role in a subset of the Rad53p-dependent checkpoint pathways controlling cell cycle progression in response to DNA damage.

Keywords: budding yeast/cell cycle/checkpoints/DNA damage/DNA primase

Introduction

Eukaryotic cells have developed a network of highly conserved surveillance mechanisms (checkpoints), ensuring that damaged chromosomes are repaired before being replicated or segregated. These mechanisms are essential for maintaining genome integrity and cell viability by delaying cell cycle progression in response to DNA damage, and several studies have linked the damage response pathways to cell cycle events (for reviews, see Hartwell and Weinert, 1989; Hartwell and Kastan, 1994; Murray, 1994; Nurse, 1994; Carr and Hoekstra, 1995; Humphrey and Enoch, 1995; Lydall and Weinert, 1996). Entry into S phase is delayed when DNA damage is induced in G_1 and, in *Saccharomyces cerevisiae*, this control is dependent on the *RAD9*, *RAD53/MEC2/SAD1/SPK1* and *RAD24* genes (Siede *et al.*, 1993, 1994; Allen *et al.*, 1994). *RAD53*, together with the *MEC1/ESR1* gene, is also required for the checkpoint which slows down the rate of DNA synthesis when DNA is damaged during S phase (Paulovich and Hartwell, 1995). Furthermore, when DNA damage is induced in G_2, cells are able to delay entry into mitosis through a surveillance mechanism, which, in *S.cerevisiae*, involves the *RAD9*, *RAD17*, *RAD24*, *RAD53*, *MEC1* and *MEC3* gene products (Allen *et al.*, 1994; Weinert *et al.*, 1994). Finally, the budding yeast *RAD53*, *MEC1* and *POL2* genes are required to prevent entry into mitosis when DNA replication is blocked (Allen *et al.*, 1994; Weinert *et al.*, 1994; Navas *et al.*, 1995).

Although it has been demonstrated that the cell cycle checkpoints are genetically controlled, the roles of the different checkpoint proteins and the final targets of the signal transduction pathways leading to cell cycle delay as a consequence of DNA damage or replication block are still unknown. The target of the checkpoint responding to DNA damage in G_2 may be factors controlling execution of mitosis, while components of the replication apparatus may act as sensors of DNA damage and stalled replication forks, and/or as targets of the checkpoint mechanisms controlling entry and progression through S phase. The involvement of replication proteins in cell cycle checkpoints is supported by the finding that, in fission yeast, the *cdc18*$^+$, *cut5*$^+$ and *cdt1*$^+$ genes are required not only for initiation of DNA synthesis, but also for the surveillance mechanisms preventing cells from entering mitosis when either arrested or delayed in S phase (Kelly *et al.*, 1993; Hofmann and Beach, 1994; Saka *et al.*, 1994). Moreover, fission yeast DNA polymerases α and δ and budding yeast DNA polymerase ε recently have been implicated in the same mechanism (Araki *et al.*, 1995; D'Urso *et al.*, 1995; Francesconi *et al.*, 1995; Navas *et al.*, 1995). Finally, the *Schizosaccharomyces pombe cds1*$^+$ gene, the homolog of *RAD53*, has been identified as a multicopy suppressor of a temperature-sensitive (ts) mutant in the DNA polymerase α gene (Murakami and Okayama, 1995).

The highly conserved DNA polymerase α–primase (pol α–primase) complex is required for both the initiation and elongation steps of DNA replication and is the target of different regulatory mechanisms during the cell cycle (Johnston and Lowndes, 1992; Campbell, 1993; Muzi Falconi *et al.*, 1993; Foiani *et al.*, 1995; Ferrari *et al.*, 1996). The genes encoding the four subunits of the budding yeast pol α–primase complex have been cloned, and several mutants have been produced and characterized (Lucchini *et al.*, 1987, 1990; Francesconi *et al.*, 1991; Longhese *et al.*, 1993; Foiani *et al.*, 1994). None of them showed any sensitivity to DNA-damaging agents.

Here, we describe the production of several new mutations in the *PRI1* gene encoding the catalytic primase subunit of the budding yeast pol α–primase complex, and the characterization of the cell cycle defects associated with the ts *pri1-M4* mutation. The *pri1-M4* mutant is defective in responding to DNA damage in G_1/S and during S phase, and a role for DNA primase in the surveillance mechanisms controlling the rate of progres-

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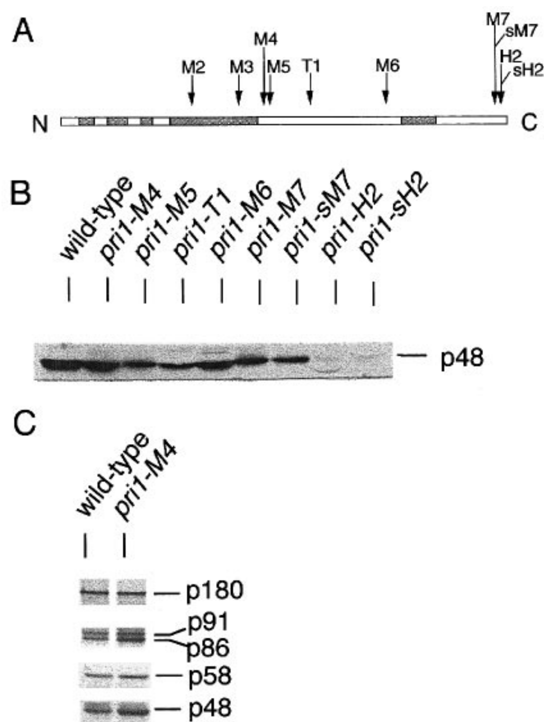

Fig. 1. Primase stability in *pri1* mutants. (**A**) A bar schematically represents the p48 polypeptide. Shaded boxes within the bar indicate conserved amino acid regions. Arrows indicate the position of the two amino acid insertions described in Materials and methods. (**B**) Twenty five µg of protein extracts prepared from the indicated strains were analyzed by Western blotting as described in Materials and methods. (**C**) A total of 3.5 mg of protein extracts from the indicated strains were immunoprecipitated with the anti-p180 y48 monoclonal antibody (Ferrari *et al.*, 1996), and analyzed by SDS–PAGE and Western blotting with specific antibodies against the pol α–primase subunits.

sion through S phase in response to DNA damage will be discussed.

Results

Mutagenesis of the PRI1 gene

We have mutagenized the *PRI1* gene carried on a centromeric plasmid by using the two-codon insertion technique (Barany, 1988). Among the obtained mutations (Figure 1A, Materials and methods), the *pri1-M4* and *pri1-T1* alleles caused a ts phenotype, *pri1-M2* and *pri1-M3* were lethal, while the other mutations did not result in any detectable phenotype (data not shown).

As shown in Figure 1B, the level of the p48 primase polypeptide was reduced dramatically in *pri1-H2* and *pri1-sH2* protein extracts, while the amount of p48 only partially decreased in *pri1-M4* extracts and pol α–primase complex formation and stability were not affected (Figure 1B and C). Shift to the restrictive temperature of *pri1-M4* mutant cells did not influence either the p48 level or the stability of the pol α–primase complex.

The pri1-M4 mutant is defective in DNA synthesis

The *pri1-M4* mutant is partially defective in DNA synthesis already at the permissive temperature. In fact, *pri1-M4* cells, exponentially growing at 25°C, were mostly budded, and fluorescence-activated cell sorter (FACS) analysis showed an accumulation of S phase cells (Figure 2A). Moreover, when *pri1-M4* cells were arrested in G_1 with α-factor, and then released from the α-factor block at permissive temperature, they were delayed in reaching G_2, although FACS analysis did not allow us to distinguish between a defect in entering S phase and a slower progression through S phase (Figure 2A).

The *pri1-M4* mutation caused a tight ts phenotype, since *pri1-M4* cells released from the α-factor block at the restrictive temperature (36°C) arrested as large-budded cells, with a single nucleus, short spindle and a 1C DNA content (Figure 2A and data not shown), suggesting that they failed to execute an early step of DNA synthesis. Finally, *pri1-M4* cells showed first cell cycle arrest (Hereford and Hartwell, 1974; Hartwell 1976), either when blocked at 36°C and then released at the permissive temperature in the presence of hydroxyurea (HU), or when first arrested in HU at 25°C and then released from the HU block at 37°C (see Materials and methods). This finding indicates that DNA primase is required for ongoing DNA synthesis, although it does not exclude the possibility that DNA primase may also play an essential function in initiation of DNA synthesis, as suggested by the results shown in Figure 2A.

The ts phenotype associated with the *pri1-M4* mutation is recessive, since both the growth rate and FACS profile of *PRI1/pri1-M4* heterozygous and *PRI1/PRI1* homozygous diploid strains were indistinguishable from each other at 36°C.

A dominant and allele-specific DNA damage sensitivity is associated with the pri1-M4 mutation

The *pri1-M4* mutant is significantly sensitive to DNA-damaging agents at the permissive temperature. In fact, when *pri1-M4* cultures were UV irradiated or treated with the alkylating agent methyl methanesulfonate (MMS), the percentage of viable cells decreased compared with the isogenic wild-type (Figure 2B and C). Moreover, the *pri1-M4* allele caused increased sensitivity to the radiomimetic drug bleomycin (data not shown).

DNA damage sensitivity was specific for the *pri1-M4* mutant, since neither different *pri1* alleles, nor mutations in the genes encoding the other subunits of the pol α–primase complex, which severely affect DNA synthesis, were more sensitive than wild-type to UV, MMS and bleomycin (data not shown).

The sensitivity to UV and MMS treatments associated with the *pri1-M4* mutation is dominant, since the *pri1-M4/PRI1* heterozygous diploid strain showed a DNA damage sensitivity comparable with that of the *pri1-M4/pri1-M4* homozygous strain (Figure 2D and E). However, DNA damage sensitivity of *pri1-M4/PRI1* heterozygous cells was similar to that of *PRI1/PRI1* homozygous cells when tested at 37°C (Figure 2F and G), probably due to inactivation of the *pri1-M4* gene product. Therefore, while the ts phenotype associated with the *pri1-M4* mutation is recessive, DNA damage sensitivity at the permissive temperature is allele specific and dominant.

Cell cycle delay in response to DNA damage during G_1 or S phase is reduced in the pri1-M4 mutant

Genetically distinguishable surveillance mechanisms are employed to delay cell cycle progression in response to

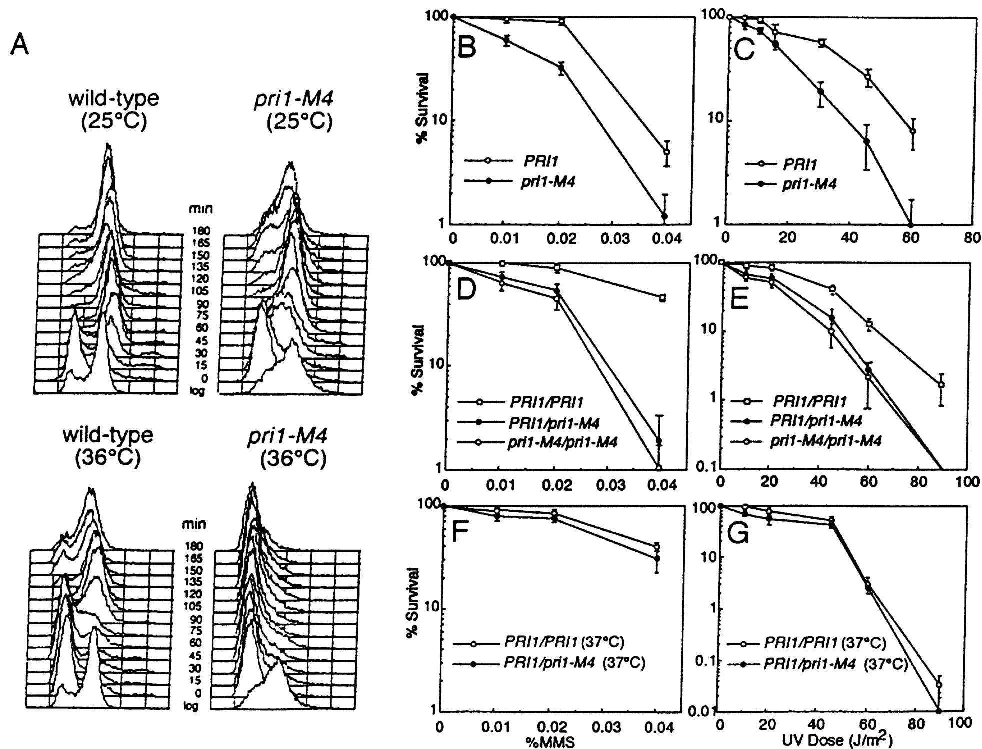

Fig. 2. *pri1-M4* is defective in an early step of DNA synthesis and is sensitive to DNA-damaging agents. (**A**) Cultures of strains K699 (wild-type) and CY387 (*pri1-M4*) logarithmically growing at 25°C (log) were synchronized by α-factor treatment (2 μg/ml) and shifted either to 25 or to 36°C at time zero after α-factor release. Samples were taken at the indicated times and analyzed by FACS. (B–G) One hundred and 1000 cells from overnight saturated YPD cultures of strains K699 (*PRI1*), CY387 (*pri1-M4*), CYd438 (*PRI1/PRI1*), CYd439 (*PRI1/pri1-M4*) and CYd524 (*pri1-M4/pri1-M4*) were either plated on YPD medium containing the indicated MMS concentrations (**B**, **D** and **F**) or UV irradiated on YPD plates at the indicated dosages (**C**, **E** and **G**). Plates were incubated at 25°C (B–E) or at 37°C (F and G) and colonies were counted after 3–4 days. Strain Cyd524 did not give rise to any colony when incubated at 37°C (F and G). Standard deviations were calculated using two to three samples. The experiments in (B–G) were performed two to four times with similar results.

DNA damage (Weinert *et al.*, 1994; Carr and Hoekstra, 1995; Friedberg *et al.*, 1995; Lydall and Weinert, 1996). Wild-type cells, UV irradiated in G_1, delay the G_1–S transition, probably to allow DNA repair (Figure 3A), while mutant strains defective in this checkpoint mechanism replicate DNA prematurely and lose cell viability (Siede *et al.*, 1993; Allen *et al.*, 1994).

In *pri1-M4* cultures released from a G_1 block after UV treatment, both bud emergence (Figure 3B) and the appearance of cells with a 2C DNA content (Figure 3A) occurred earlier when compared with the wild-type strain in the same conditions, suggesting that *pri1-M4* cells are partially defective in properly delaying cell cycle progression in response to UV irradiation during G_1. This phenotype was associated with increased cell lethality, which was almost completely prevented by holding the cells in α-factor for at least 60 min after UV irradiation (Figure 3C).

Another genetically controlled regulatory mechanism, requiring the *RAD53* and *MEC1* genes, slows the rate of S phase progression, when DNA damage occurs during DNA replication (Paulovich and Hartwell, 1995). When *pri1-M4* cultures were released from α-factor block in the presence of MMS, progression through S phase was more rapid than in wild-type (Figure 4A), and cell viability was strongly reduced (Figure 4B). Hence, the rate of progression through S phase in *pri1-M4* shows only partial, if any, reduction in response to MMS treatment and, paradoxically, this mutant allele, which is defective in DNA synthesis, replicates DNA faster than wild-type under these conditions. *pri1-M4* cells held in α-factor throughout the MMS treatment mantained a 1C DNA content and did not lose cell viability (Figure 4B), suggesting that increased cell lethality induced by DNA damage in the *pri1-M4* mutant strain is related to its faster progression through S phase.

In order to correlate *pri1-M4* DNA damage sensitivity to its intra-S checkpoint defect, we tested whether faster S phase progression of *pri1-M4* cells in the presence of MMS was also dominant. We found that *pri1-M4* [pFE139] cells, containing a centromeric plasmid carrying the wild-type *PRI1* gene, failed to properly delay cell cycle progression in the presence of MMS, similarly to what was observed in *pri1-M4* [pFE202] cells, carrying the *pri1-M4* allele on the same vector (Figure 4C). As expected, the checkpoint defect of *pri1-M4* [pFE139] cells caused an increase in cell lethality which was prevented by α-factor treatment (Figure 4D). However, both the ts phenotype (data not shown) and the mitotic cell cycle delay in the absence of MMS treatment observed in *pri1-M4* cells were abolished in *pri1-M4* [pFE139] cells (Figure 4C). Since the *pri1-M4* intra-S checkpoint defect is dominant and can be distinguished genetically from the recessive DNA synthesis defect, the inability to delay properly the rate of S phase progression in response to DNA damage is unlikely to be related to a general

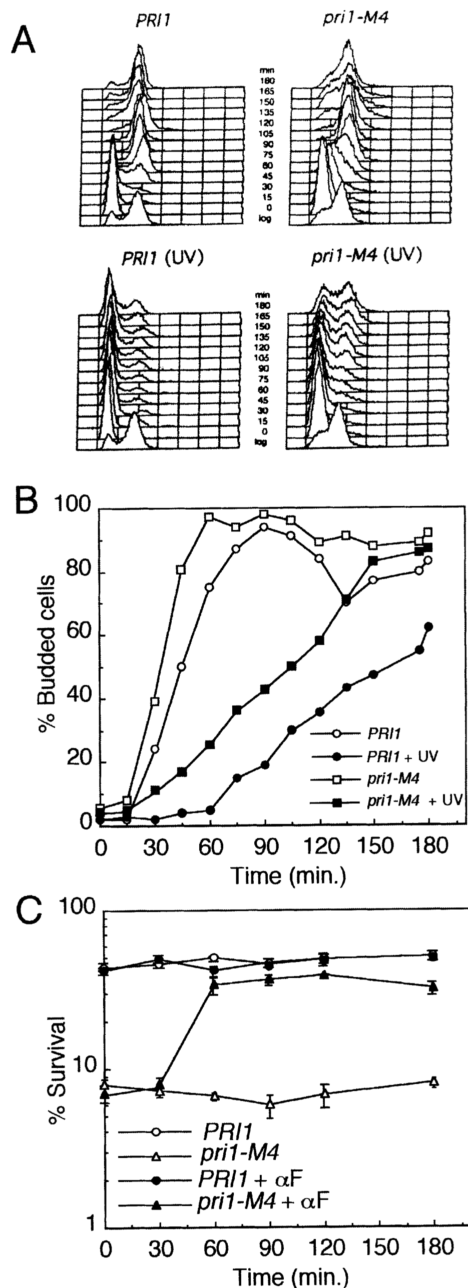

Fig. 3. The *pri1-M4* mutant is defective in responding to UV irradiation during G_1. Cultures of strains K699 (*PRI1*) and CY387 (*pri1-M4*), logarithmically growing at 25°C (log), were pre-synchronized with 2 μg/ml of α-factor, spread on YPD plates and then UV irradiated as described in Materials and methods. (**A**) Samples taken at the indicated times after α-factor release were analyzed by FACS. *PRI1* cells were able to recover from the UV-induced cell cycle delay after 4 h, and cell number after UV irradiation did not increase from time 0 to 180 min. (**B**) The percentage of budded cells monitored at the indicated times in both unirradiated and irradiated (+ UV) cultures. Wild-type and *pri1-M4* cultures mock UV irradiated and treated again with α-factor remained unbudded and with a 1C DNA content throughout the experiment (180 min) (data not shown). (**C**) Cell survival was measured at the indicated times, as described in Materials and methods. + αF indicates cell cultures kept in 5 μg/ml of α-factor to maintain the G_1 block after UV irradiation. Standard deviations were calculated by using samples from two independent experiments in which the FACS profiles were similar to that shown in (A).

disturbance of the whole replication apparatus. This conclusion is supported further by the finding that the *pri2-1* mutant, which is altered in the p58 subunit of the pol α–primase complex, failed to execute an early step of DNA synthesis after shift to the restrictive temperature (Figure 5A). Nevertheless, the *pri2-1* mutation did not alter the intra-S checkpoint at the permissive temperature, but rather caused a slower progression through S phase in the presence of MMS compared with wild-type (Figure 5B). This behavior is likely to be due to a proficient intra-S checkpoint superimposed on a DNA replication defect. Accordingly, the cell viability of *pri2-1* cells in the presence of MMS was identical to wild-type (Figure 5C).

pri1-M4 cells are not defective in delaying mitosis in response to DNA damage or HU treatment

Wild-type cells respond to DNA damage in G_2 by delaying entry into mitosis through a regulatory pathway involving the *RAD9*, *RAD17*, *RAD24*, *RAD53*, *MEC1*, *MEC3* and *PDS1/ESP2* gene products (Allen *et al.*, 1994; Weinert *et al.*, 1994; Yamamoto *et al.*, 1996). As shown in Figure 6, *pri1-M4* mutant cells, UV irradiated in G_2, properly delayed entry into mitosis, while a *rad9*Δ strain failed to restrain mitotic entry in response to DNA damage. Therefore, *pri1-M4* cells are proficient in the G_2 DNA damage checkpoint.

Another checkpoint links entry into mitosis to the completion of the preceding S phase. This interdependency is lost in *rad53*, *mec1* and *pol2* mutants, which die in the presence of HU with elongated mitotic spindles and divided nuclei (Allen *et al.*, 1994; Weinert *et al.*, 1994; Navas *et al.*, 1995). Cell viability of logarithmically growing or α-factor pre-synchronized *pri1-M4* cultures was not affected by treatment with 0.2 M HU and, accordingly, cells arrested as large budded cells with a single undivided nucleus, short spindles and an S phase DNA content (data not shown). These data suggest that *pri1-M4* cells can properly delay entry into mitosis when DNA is not replicated completely.

Genetic interactions between pri1-M4 and checkpoint-defective mutants

The *MEC1* and *RAD53* genes encode essential proteins involved in the signal transduction pathway that is activated in response to DNA damage in G_1, S and G_2 (Allen *et al.*, 1994; Kato and Ogawa, 1994; Weinert *et al.*, 1994). The *MEC3* gene is not essential, but *mec3* mutants are defective in their ability to delay cell cycle progression in response to DNA damage (Weinert *et al.*, 1994; Longhese *et al.*, 1996). Combination of the *pri1-M4* allele with mutations in *MEC1* (*esr1-1*, *mec1-1*), *RAD53* (*sad1-1*, *mec2-1*) and *MEC3* (*mec3-1*) genes resulted in cell lethality. In fact, we were unable to recover any viable double mutants at the permissive temperature after sporulation of the appropriate heterozygous diploids and analysis of 16, 36, 24, 12 and 17 tetrads, respectively, for each strain. Since we recovered all the other expected genotypes, we infer that the double mutants were formed during meiosis, but were inviable.

The pathway leading to Rad53p phosphorylation is proficient in the pri1-M4 mutant

Rad53p is an essential protein kinase that plays a pivotal role in the pathway delaying cell cycle progression when

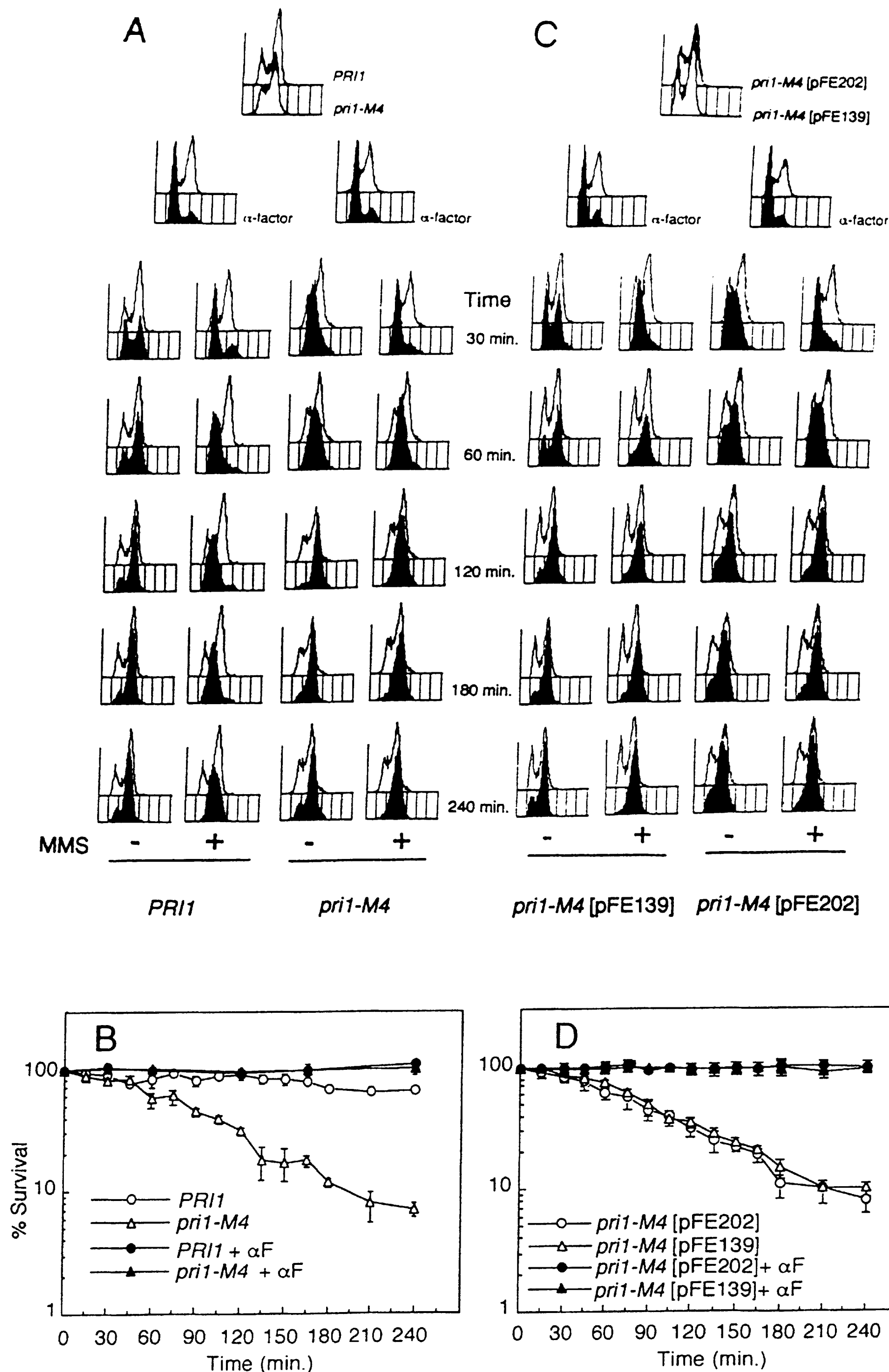

Fig. 4. The *pri1-M4* mutant is defective in slowing down S phase progression in the presence of DNA damage. (**A**) and (**C**) Log-phase (log) cultures of strains K699 (*PRI1*), CY387 (*pri1-M4*), CY387 transformed with the pFE139 centromeric plasmid carrying the wild-type *PRI1* gene (*pri1-M4*[pFE139]) and CY387 transformed with the pFE202 centromeric plasmid carrying the *pri1-M4* allele (*pri1-M4*[pFE202]) were pre-synchronized by α-factor treatment and released in YPD with or without 0.02% MMS. Samples were taken at the indicated times for FACS analysis (black histograms). Overlayed histograms represent the cell cycle distributions of the asynchronous cultures before α-factor treatment. The experiments shown in (A) and (C) were performed three times and twice, respectively, with similar results. FACS profiles of strain K699 transformed with the centromeric plasmid pFE139 were indistinguishable from that shown for the non-transformed K699 strain. (**B**) and (**D**) Cell survival was measured at the indicated times, as described in Materials and methods. + αF indicates cell cultures kept in 5 μg/ml of α-factor throughout the MMS treatment to maintain the G_1 block. Standard deviations were calculated by using samples from two independent experiments in which the FACS profiles were similar to that shown in (A) and (C).

DNA is damaged or replication is not complete (Zheng *et al.*, 1993; Allen *et al.*, 1994; Weinert *et al.*, 1994). Rad53p is phosphorylated in *trans* by a Mec1p- and Mec3p-dependent mechanism in response to DNA damage (Sanchez *et al.*, 1996; Sun *et al.*, 1996). A Mec1p-dependent mechanism causes Rad53p phosphorylation during S phase under conditions perturbing proper cell cycle progression (Sun *et al.*, 1996), but this is not observed in logarithmically growing wild-type cells or during S phase of α-factor pre-synchronized cell cultures (Figure 7B, and Sun *et al.*, 1996).

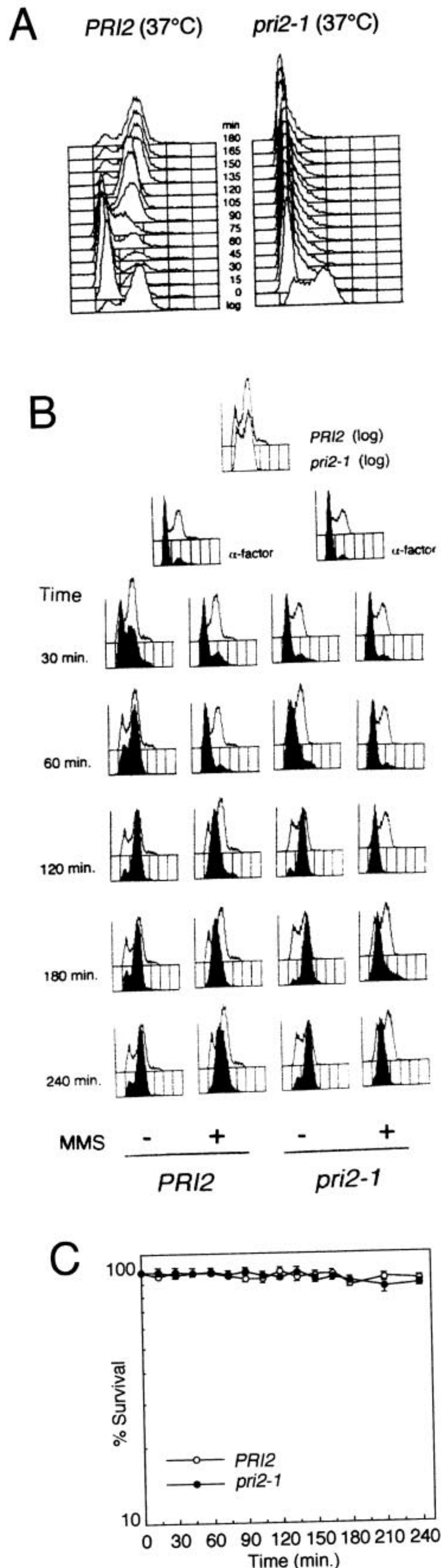

Fig. 5. The intra-S checkpoint is proficient in the ts *pri2-1* mutant. (**A**) Cultures of strains K699 (*PRI2*) and its isogenic derivative (*pri2-1*), logarithmically growing at 25°C (log) were synchronized by α-factor treatment (2 μg/ml) and shifted to 37°C at time zero, after α-factor release. Samples were taken at the indicated times and analyzed by FACS. (**B**) Cultures of strain K699 (*PRI2*) and its isogenic derivative (our unpublished data), in which the *PRI2* gene was replaced with the *pri2-1* allele (*pri2-1*) (Francesconi *et al.*, 1991), logarithmically growing at 25°C (log), were synchronized by α-factor treatment and released at 25°C in liquid medium with or without 0.02% MMS. Samples were taken at the indicated times for FACS analysis (black histograms). Overlayed histograms represent the cell cycle distributions of the asynchronous cultures before α-factor treatment. (**C**) Cell survival of the indicated strains in the presence of MMS was measured at the indicated times, as described in Materials and methods.

Since synthetic lethality of *pri1-M4 sad1-1* and *pri1-M4 mec2-1* double mutants suggests that DNA primase and Rad53p functionally interact, we made an attempt to order the relative function of Rad53p and DNA primase by testing Rad53p phosphorylation in *pri1-M4* cells under different conditions. As shown in Figure 7A, Rad53p was phosphorylated in HU- or MMS-treated *PRI1* and *pri1-M4* cells, as indicated by the appearance of isoforms with lower electrophoretic mobility. Since HU and MMS treatments result in accumulation of S phase cells, we tested whether Rad53p phosphorylation was still occurring when cells were held in α-factor during MMS treatment. As shown in Figure 7A, Rad53p was still phosphorylated under this condition, indicating that this post-translational modification was related specifically to DNA damage response in G_1-arrested cells. This finding suggests that the cascade of events leading to Rad53p phosphorylation caused by genotoxic agents is proficient in *pri1-M4* cells.

Moreover, the *RAD53* pathway seems to be activated also in response to cell cycle perturbations due to a defective DNA primase. In fact, as shown in Figure 7A, Rad53p phosphorylation is also observed in logarithmically growing *pri1-M4* mutant cultures, while it cannot be detected in wild-type cells. In *pri1-M4* mutant cells pre-synchronized by α-factor treatment, Rad53p phosphorylation was detected already 15 min after α-factor release (Figure 7B). It is likely that S phase is already started 15 min after α-factor release, although FACS analysis only revealed a slower S phase progression in the mutant culture (Figure 7B). In this view, a defective DNA primase will cause the accumulation of replication intermediates which will activate the checkpoint pathway leading to Rad53p phosphorylation. This assumption is substantiated by the finding that Rad53p phosphorylation is not detected in logarithmically growing *PRI1/pri1-M4* heterozygous cells (Figure 7C), in agreement with the observation that the *pri1-M4* replication defect is recessive. Conversely, the same heterozygous strain, which is checkpoint defective (see Figure 4), is still able to phosphorylate Rad53p in response to HU or MMS treatment (Figure 7C).

It has been shown that transcription of DNA damage-inducible genes requires a functional Rad53p-dependent pathway (Zhou and Elledge, 1993; Allen *et al.*, 1994; see Figure 9A). To test whether this pathway was active in *pri1-M4* mutant cells, we measured the expression of the DNA damage-inducible gene *RNR2* by assaying β-galactosidase activity in extracts from *pri1-M4* cells carrying a *RNR2–lacZ* fusion gene integrated at the *LEU2* locus. As shown in Figure 7A, *pri1-M4* mutant cells were

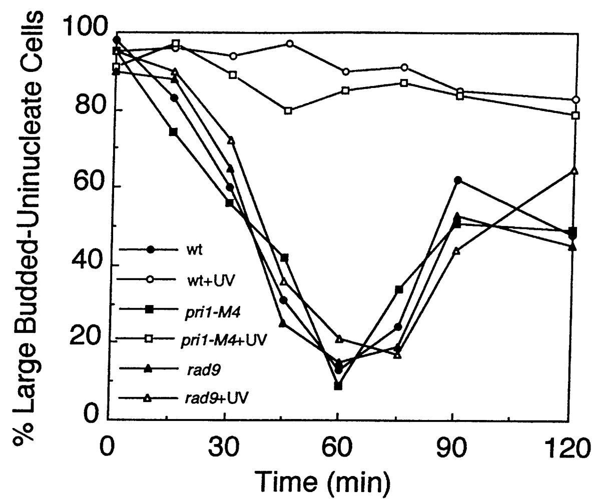

Fig. 6. *pri1-M4* is able to delay mitosis in response to G2-induced DNA damage. Strains K699 (*PRI1*), CY387 (*pri1-M4*) and CY427 (*rad9*) were arrested in G2/M with nocodazole and UV irradiated (see Materials and methods). Irradiated (+UV) and unirradiated samples were resuspended in fresh medium and the percentage of large budded uninucleate cells was scored by DAPI staining as described in Materials and methods. The experiment with the different strains was repeated two to three times with similar results.

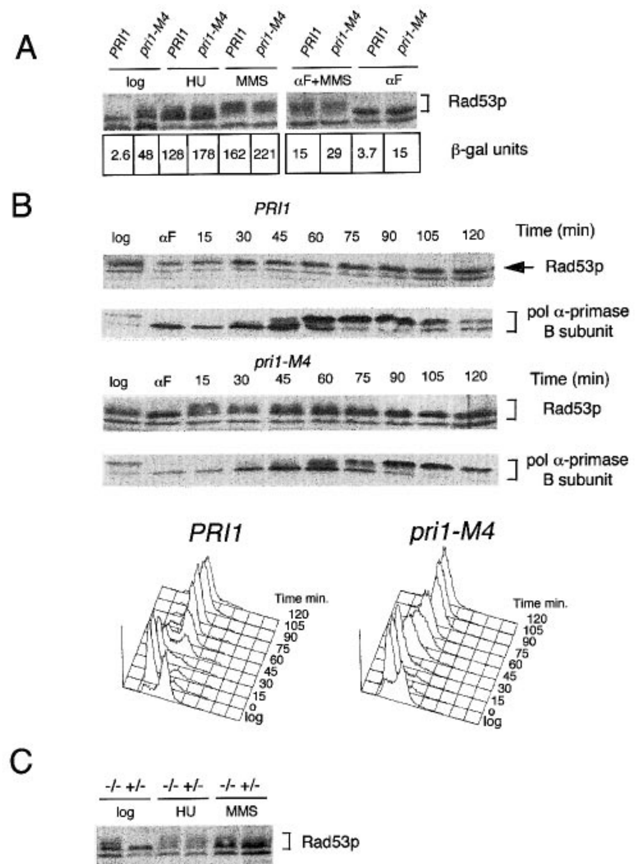

Fig. 7. The *pri1-M4* mutation does not interfere with Rad53p phosphorylation. (**A**) Total protein extracts were prepared by the TCA procedure (Materials and methods) from log-phase (log) cultures of strains K699 (*PRI1*) and CY387 (*pri1-M4*), or from cultures of the same strains incubated at 25°C for 4 h in YPD medium containing 0.2 M HU (HU) or 0.02% MMS (MMS). Extracts were also prepared from cultures of the same strains held for 4 h in 5 μg/ml of α-factor with (αF + MMS) or without (αF) 0.02% MMS after α-factor pre-synchronization. SDS–PAGE, followed by Western blotting with anti-Rad53p antibodies (Sun *et al.*, 1996), was performed as described in Materials and methods. The bands corresponding to phosphorylated and unphosphorylated Rad53p are indicated by a bracket. A protein species, migrating slightly faster than unphosphorylated Rad53p, cross-reacts with anti-Rad53p antibodies. β-Galactosidase activity in extracts prepared from strains CY1066 (*PRI1*) and CY1068 (*pri1-M4*), carrying an integrated *RNR2–lacZ* fusion gene and treated as described above, was assayed as previously described (Lucchini *et al.*, 1984). The reported β-gal units are averages of results obtained from assays on two to three independent extracts, and standard error was always <20%. (**B**) Log-phase (log) cultures of K699 (*PRI1*) and CY387 (*pri1-M4*) were arrested in G_1 by α-factor treatment (αF) and released from the α-factor block in YPD. After α-factor release, samples were taken at the indicated times and analyzed by FACS and by Western blotting using antibodies against Rad53p and pol α–primase B subunit which is phosphorylated during S phase (Foiani *et al.*, 1994, 1995). Brackets indicate phosphorylated and unphosphorylated forms of Rad53p and pol α–primase B subunit. (**C**) Total protein extracts were prepared as described in (A) from log-phase (log) cultures of strain CY387 transformed with the pFE139 centromeric plasmid carrying the wild-type *PRI1* gene (–/+) and CY387 transformed with the pFE202 centromeric plasmid carrying the *pri1-M4* allele (–/–), or from cultures of the same strains incubated at 25°C for 4 h in YPD medium containing 0.2 M HU (HU) or 0.02% MMS (MMS). Western blotting with anti-Rad53 antibodies was performed as described in (A).

still able to induce the expression of the *RNR2–lacZ* fusion in response to genotoxic agents, even when the MMS treatment was performed in α-factor-arrested cells. When both wild-type and *pri1-M4* cells were held in α-factor, *RNR2–lacZ* induction was much lower than in cycling cells. It is possible that cycling cells are more efficient in inducing *RNR2–lacZ* expression during the S phase, although Rad53p phosphorylation seems to be similar in cycling and α-factor-arrested cells in response to DNA-damaging agents. Further experiments will be required to address the apparent uncoupling between the efficiency of Rad53p phosphorylation and *RNR2–lacZ* induction.

The expression of the *RNR2–lacZ* fusion in untreated *pri1-M4* cells was higher than in the wild-type, and a similar phenotype was found to be associated with mutations, called *crt*, which cause constitutive expression of DNA damage-inducible genes (Zhou and Elledge, 1992). These data are in agreement with the previous suggestion that a defective DNA primase leads to accumulation of DNA lesions, resulting in activation of *RNR2* expression through the Rad53p-dependent pathway, which is proficient in *pri1-M4* cells. Accordingly, we found that the *pri1-M4*-associated Crt$^-$ phenotype is recessive, as is the DNA replication defect (data not shown).

The genetic interactions discussed in the previous section suggest that Rad53p and DNA primase act in the same pathway, and the analysis of Rad53p phosphorylation in *pri1-M4* cells and in the *PRI1/pri1-M4* heterozygous strain further suggests that DNA primase is not involved in the cascade of events leading to Rad53p phosphorylation in response to DNA damage.

Since it has been shown recently that *RAD53* overexpression delays bud emergence and S phase entry (Sun *et al.*, 1996), we compared the effect of *RAD53* overexpression in wild-type and *pri1-M4* cells. As shown in Figure 8A and B, the *pri1-M4* mutation partially counteracts the effect of *RAD53* overexpression on both budding kinetics and timing of S phase entry, suggesting that DNA primase might act downstream of *RAD53*. However, we did not observe any mobility shift of the p48 DNA primase

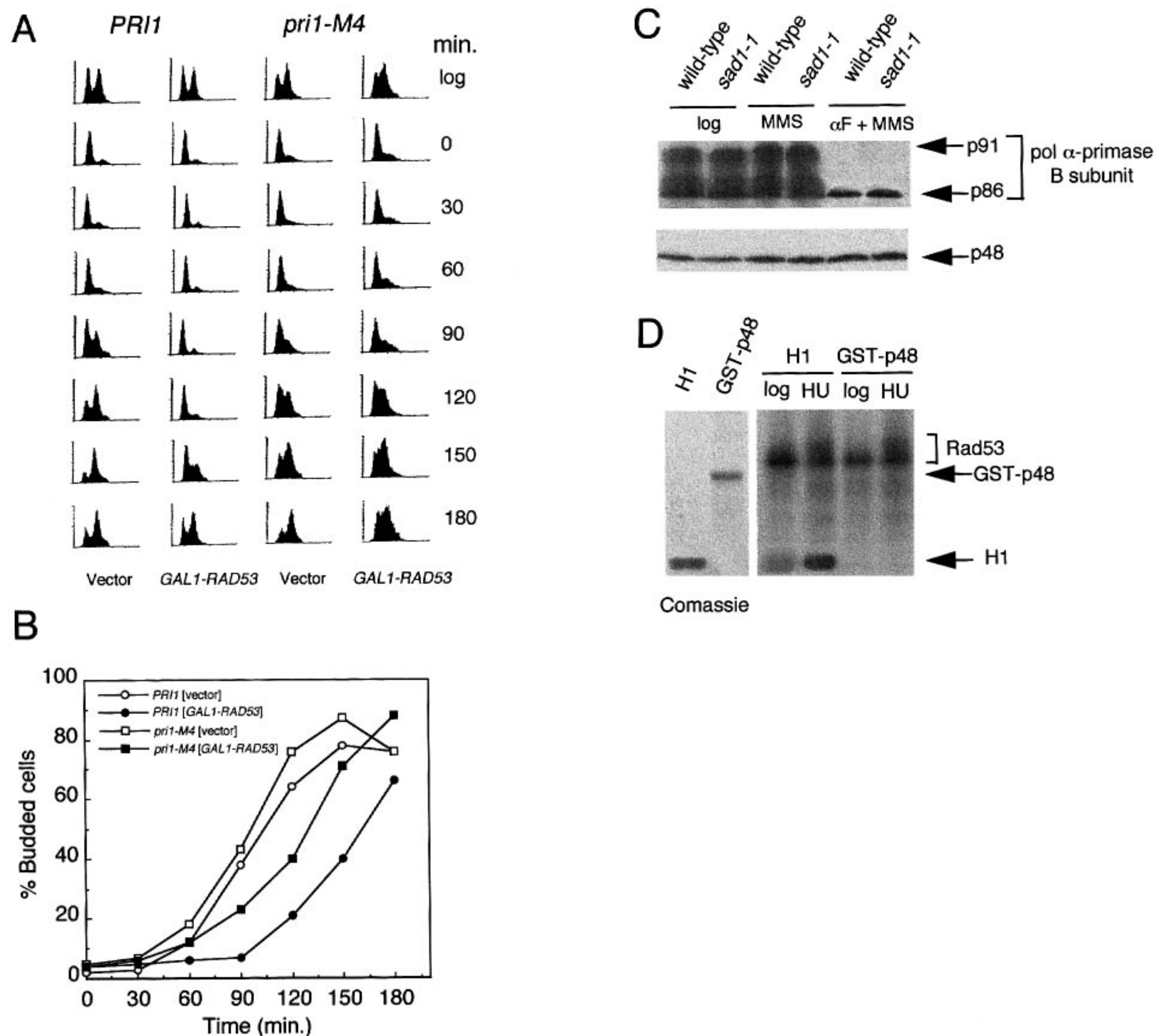

Fig. 8. The *pri1-M4* mutation counteracts the cell cycle delay caused by Rad53p overexpression. (**A**) Strains K699 (*PRI1*) and CY387 (*pri1-M4*) were transformed with plasmids pNB187 (Vector) or pNB187-*SPK1* (*GAL1-RAD53*). Transformants were grown in SD medium containing 2% raffinose (log), synchronized by α-factor treatment and released from the α-factor block at 25°C in SD medium containing 2% raffinose and 2% galactose. Samples for FACS analysis were taken at the indicated times after α-factor release (time 0). (**B**) The percentage of budded cells was monitored, at the indicated times, in the same strains described in (A). (**C**) Total protein extracts were prepared by the TCA procedure (Materials and methods) from cultures of strains Y300 (wild-type) and Y301 (*sad1-1*) logarithmically growing in YPD (log), growing for 4 h in YPD containing 0.02% MMS (MMS), or held in G_1 for 4 h by α-factor treatment in the presence of 0.02 MMS (αF + MMS). Proteins were separated on low cross-linking SDS–polyacrylamide gels as described in Materials and methods, and immunoreactive polypeptides were visualized on Western blots with anti-B subunit and anti-p48 antibodies. (**D**) *In vitro* phosphorylation of histone H1 and of a GST–p48 fusion protein was carried out as described in Sun *et al.* (1996). The GST–p48 fusion protein was purified by affinity chromatography (Mitchell *et al.*, 1993) from yeast extracts prepared from a strain expressing a GST–p48 fusion protein.

subunit in wild-type and in *rad53* mutant (*sad1-1*) cell extracts prepared from untreated or MMS-treated cells, even in conditions which magnify the difference in electrophoretic mobility between the unphosphorylated p86 and the hyperphosphorylated p91 isoforms of the pol α–primase B subunit (Figure 8C). Moreover, immunoprecipitated Rad53p is not able to phosphorylate a GST–p48 fusion *in vitro*, while Rad53p is able to phosphorylate histone H1 under the same conditions (Figure 8D). Therefore, although DNA primase might act downstream of Rad53p, it does not seem to be a direct substrate of Rad53p kinase.

Discussion

Role of DNA primase in DNA synthesis

The p48 subunit of the budding yeast pol α–primase complex is sufficient for DNA primase activity *in vitro* (Santocanale *et al.*, 1993), and previous characterization of *pri1* mutants established that p48 is essential for cell viability and DNA replication *in vivo* (Francesconi *et al.*, 1991; Longhese *et al.*, 1993).

Production of new *pri1* mutants has allowed the identification of the tight ts *pri1-M4* allele. Reciprocal shift experiments and FACS analysis performed on *pri1-M4* cells showed that p48 is required for ongoing DNA

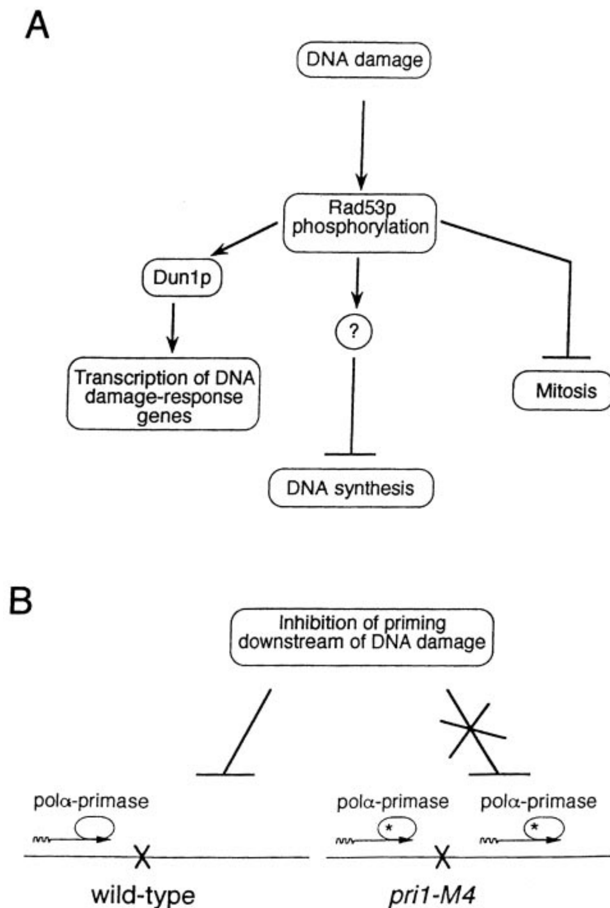

Fig. 9. Model for DNA primase response to DNA damage. (**A**) The roles of Rad53p in response to DNA damage are represented schematically. Rad53p is phosphorylated in DNA damage conditions (Sanchez *et al.*, 1996; Sun *et al.*, 1996) and is required for activation of the Dun1p-dependent pathway that leads to transcription of DNA damage-inducible genes (Zhou and Elledge, 1993). Rad53p is also required in order to delay the cell cycle by negatively regulating progression through S phase (Paulovich and Hartwell, 1995) and entry into mitosis (Allen *et al.*, 1994; Weinert *et al.*, 1994). The question mark indicates as yet unidentified factor(s), possibly mediating the cascade from Rad53p to the replication machinery. (**B**) Wild-type cells, experiencing DNA damage while progressing through S phase, slow down DNA synthesis through the action of the Rad53p-dependent checkpoint pathway, which negatively regulates the priming activity associated with the pol α–primase complex, thus preventing initiation of DNA synthesis downstream of the lesions. In *pri1-M4* cells, the primase subunit of the pol α–primase complex is unable to respond properly to this inhibitory signal and synthesizes RNA primers downstream of the damage, allowing DNA synthesis to occur. The arrows represent the elongation products of newly synthesized RNA primers (wavy lines), and the asterisk indicates the mutated primase subunit.

synthesis. Furthermore, the observation that *pri1-M4* cells arrested with a 1C DNA content, after α-factor release at the restrictive temperature, indicates that the bulk of DNA synthesis cannot be performed in this mutant at the non-permissive temperature. Since FACS analysis is not sensitive enough to distinguish between a defect in initiation of DNA replication and an impairment in some early step of DNA elongation, further biochemical characterization will be required to establish firmly a direct role for DNA primase in initiation of DNA replication at an origin *in vivo*.

DNA damage sensitivity of the pri1-M4 mutant

The previously characterized *pri1* mutants did not show any increased sensitivity to DNA-damaging agents compared with wild-type, although they were defective in DNA synthesis and caused enhanced rates of mitotic intrachromosomal recombination and mutation, probably due to the accumulation of DNA lesions (Francesconi *et al.*, 1991; Longhese *et al.*, 1993). The *pri1-M4* mutant, besides being ts and defective in DNA synthesis, is also sensitive to UV radiation and MMS treatment. The ts phenotype is recessive, while DNA damage sensitivity is dominant and they are, therefore, genetically distinguishable.

DNA damage sensitivity can be due to different causes, such as defective DNA repair, inability to induce transcription of DNA damage-inducible genes or defective checkpoint mechanisms. It is unlikely that DNA primase plays any direct role in DNA repair since, with the exception of the *pri1-M4* allele, none of the alleles so far identified in the *PRI1* gene or in the genes encoding the other subunits of the pol α–primase complex exhibits DNA damage sensitivity (Lucchini *et al.*, 1990; Francesconi *et al.*, 1991; Longhese *et al.*, 1993; Foiani *et al.*, 1994). Furthermore, gap filling repair synthesis does not require RNA primer synthesis.

DNA damage, as well as inhibition of DNA synthesis, results in the transcriptional activation of DNA damage-inducible genes. Mutations in *dun* genes cause inability to induce this transcriptional response (Zhou and Elledge, 1993; Navas *et al.*, 1995), while mutations in *crt* genes cause constitutive expression of DNA damage-inducible genes (Zhou and Elledge, 1992). The *pri1-M4* mutant does not show a Dun phenotype, but rather behaves like *crt* mutants and, interestingly, the *crt5-262* mutation is allelic to the *POL1* gene, encoding the large subunit of the pol α–primase complex (Zhou and Elledge, 1992). This observation, together with the hyper-recombination and mutator phenotype associated with other *pri1*, *pri2* and *pol1* alleles (Lucchini *et al.*, 1990; Longhese *et al.*, 1993), suggests that a defective pol α–primase complex might cause the accumulation of DNA damage signals, which result in activation of DNA damage-inducible genes (see Figure 9A).

The pri1-M4 mutant is defective in properly delaying cell cycle progression in response to DNA damage

Eukaryotic cells minimize the consequence of DNA damage by activating a network of checkpoints, whose function is to delay cell cycle progression, probably to provide sufficient time for DNA repair. Mutations affecting the components of these surveillance mechanisms cause increased sensitivity to genotoxic agents. We found that *pri1-M4* cells properly restrain entry into mitosis when DNA is damaged in G_2 or DNA replication is blocked by HU treatment, indicating that the G_2–M DNA damage checkpoint and the mechanisms coupling completion of DNA replication to entry into mitosis are proficient. Conversely, the *pri1-M4* mutant fails to delay properly bud emergence and entry into S phase after UV irradiation, and to slow down the rate of DNA synthesis in the presence of MMS with a concomitant increase in cell lethality. Therefore, this mutation specifically affects only

a subset of the checkpoint pathways, and DNA damage sensitivity of the *pri1-M4* mutant may be related to its failure to delay properly S phase entry and progression in response to genotoxic agents. This is an apparent paradox: in fact, *pri1-M4* is defective in DNA synthesis at the permissive temperature in the absence of DNA-damaging agents, while the same mutant proceeds faster than wild-type through S phase in the presence of MMS. Therefore, at the permissive temperature, the partially defective *pri1-M4* gene product is still capable of carrying out DNA synthesis and probably fails to respond properly to a regulatory mechanism which is required to inhibit G_1–S transition and S phase progression in the presence of DNA damage. While a role for DNA primase in connecting DNA damage response to DNA replication is reasonable, a direct involvement of DNA primase in the budding pathway can hardly be envisaged. It is more likely that the failure of *pri1-M4* cells to delay properly bud emergenge in response to UV irradiation in G_1 is a consequence of premature entry into S phase, which then results in the activation of the budding pathway.

A possible role for DNA primase in linking DNA damage response to DNA replication

Different types of DNA damage are likely to be detected by several sensors, and the generated signal is then transduced through the Rad53p-dependent pathway in order to delay cell cycle progression and to activate transcription of DNA damage-inducible genes (Allen *et al.*, 1994; Weinert *et al.*, 1994; Navas *et al.*, 1995; Paulovich and Hartwell, 1995; Sanchez *et al.*, 1996; Sun *et al.*, 1996) (Figure 9A). It has been suggested recently that the slowing down of S phase in the presence of DNA damage, which is genetically controlled at least by the *MEC1* and *RAD53* genes, must target some component(s) of the DNA replication machinery (Paulovich and Hartwell, 1995). The Rad53p protein kinase is phosphorylated in *trans* by a Mec3p- and Mec1p-dependent mechanism in response to DNA damage (Sanchez *et al.*, 1996; Sun *et al.*, 1996), suggesting that Rad53p is an intermediate component of the signal transduction pathway coupling DNA damage to cell cycle arrest (Figure 9A).

Although further studies will be required to establish firmly the order of relative functions of *RAD53* and *PRI1*, the following observations suggest that DNA primase acts downstream of *RAD53*: (i) the pathways leading to Rad53p phosphorylation as a consequence of HU and MMS treatment are proficient in both *pri1-M4* and *PRI1/pri1-M4* cells; (ii) the *pri1-M4* mutation counteracts the cell cycle delay caused by *RAD53* overexpression; and (iii) both the *DUN1*-dependent pathway and the checkpoint preventing entry into mitosis in response to DNA damage are functional in *pri1-M4* cells. However, the results presented in Figure 8 indicate that DNA primase is not a direct substrate of Rad53p kinase and, therefore, we must assume that, if DNA primase acts downstream of *RAD53*, then other factor(s) might mediate the inhibitory signal from Rad53p to DNA primase in response to DNA damage.

Since the intra-S checkpoint (Paulovich and Hartwell, 1995) is dependent on the *MEC1*, *RAD53* and *PRI1* genes, and DNA primase seems to act downstream of Mec1p and Rad53p, we propose that this pathway might lead to inhibition of DNA primase, preventing priming downstream of the damage (Figure 9B). This model is consistent with the biochemical properties of DNA primase, which is a highly distributive enzyme with the unique property of providing the RNA primers required to initiate DNA synthesis (Kornberg and Baker, 1992). Moreover, it is well known that, although many lesions block DNA polymerases *in vitro*, cells are still able to synthesize DNA (reviewed by Naegeli, 1994). Therefore, DNA primase activity might be required to bypass a DNA lesion in order to resume DNA synthesis downstream of the damage (Figure 9B). A mechanism analogous to that required to bypass a DNA lesion occurs in *Escherichia coli* to reconstitute rolling circle synthesis, and depends on priming proteins (Allen *et al.*, 1993). The observation that the *pri1-M4* DNA damage sensitivity and the intra-S checkpoint defect are dominant further supports the hypothesis that the *pri1-M4* mutant primase fails to sense the inhibitory signal and resumes DNA synthesis downstream of the lesion. A similar mechanism might also explain the G_1–S checkpoint defect of *pri1-M4* cells. In fact, since all the genes controlling the intra-S checkpoint analyzed so far are also required to delay G_1–S transition in response to DNA damage (Lydall and Weinert, 1996; Longhese *et al.*, 1996), and since primer formation is essential to initiate DNA synthesis, it is tempting to speculate that a failure of DNA primase to respond to checkpoint inhibitory signals might also lead to premature entry into S phase.

Materials and methods

Plasmids

Plasmid pFE139 contains the 2449 bp *Pst*I–*Sac*I *PRI1* genomic fragment cloned in plasmid YCplac22 (Gietz and Sugino, 1988). Plasmid pFE5 contains the blunted 1920 bp *Nru*I–*Sac*I *PRI1* genomic fragment cloned into the *Nru*I site of plasmid YCp50 (Rose *et al.*, 1990). pLAN2 is a *ARS1 TRP1 CEN6* plasmid carrying the *PRI1* gene (Francesconi *et al.*, 1991). Plasmids pFE202 and pFE299 contain the 2449 bp *Pst*I–*Sac*I genomic fragment carrying the *pri1-M4* mutation, cloned, respectively, in plasmids YCplac22 and YIplac211 (Gietz and Sugino, 1988). The *Bgl*II-linearized pFE299 plasmid has been used to replace the chromosomal copy of *PRI1* in different genetic backgrounds by the two-step procedure (Rothstein, 1991). Plasmid pNB187-*SPK1* (Sun *et al.*, 1996) is a pNB187 derivative plasmid in which *RAD53/SPK1* expression is driven by a *GAL1*-inducible promoter. Plasmid p0-1Kpn, carrying the *RNR2–lacZ* fusion cloned into the pSZX vector (Hurd and Roberts, 1989), was provided by M.Fasullo (Loyola University, Chicago). Plasmid pRR330 carrying the *rad9*Δ cassette was provided by L.Prakash (University of Texas, Dallas). Plasmid pFE302 is a pEG(KT) derivative (Mitchell *et al.*, 1993) in which the *PRI1* coding region has been fused to an inducible *GAL1–GST* gene, and this plasmid is able to complement a lethal disruption of the *PRI1* gene.

Yeast strains

Strains K699, *MAT***a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3* and K700, *MAT*α *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3* are isogenic and were provided by K.Nasmyth (I.M.P., Vienna). Unless otherwise stated, all yeast strains used in this work are isogenic to K699. Strain CG378, *MAT***a** *ade5-7 leu2-3,112 ura3-52 trp1-289 can1* was from L.H.Johnston (NIMR, London). Strain YLAN *MAT***a** Δ*pri1 ura3-52 trp1* [pLAN2] is a L1156 derivative carrying a deletion of the *PRI1* chromosomal locus complemented by the *TRP1 CEN6* pLAN2 plasmid (Francesconi *et al.*, 1991). Strain CY124 is a YLAN derivative where the pLAN2 plasmid has been substituted by the *URA3 CEN4* pFE5 plasmid. The *PRI1* chromosomal copy has been replaced with the *pri1-M4* allele in strains K699, K700 and CG378 to originate, respectively, strains CY387, CY522 and CY399. Strains CY1066 and CY1068 are, respectively, K699 and CY387 derivatives containing one copy of the p0-1Kpn plasmid integrated at the *LEU2* locus, and were obtained as described in Hurd and Roberts (1989). Strain CY427 is a K699 derivative containing the *rad9::URA3* disruption, obtained by transformation with

the 7100 bp *Sal*I–*Eco*RI fragment of plasmid pRR330 (Schiestl *et al.*, 1989). In all cases, correct replacements and plasmid integrations were verified by Southern blotting. Strain K700 was crossed to strains CG378 and CY399 to obtain, respectively, diploid strains CYd438 and CYd439, while strain CY522 was crossed to strain CY399 to give rise to diploid strain CYd524. Strain Y301, *MAT***a** *sad1-1 can1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1* and its isogenic *RAD53/SAD1* strain (Y300) were provided by S.J.Elledge (Baylor College, Houston). Strains TWY308, *MAT*α *mec1-1 ura3 trp1*, TWY312, *MATa mec2-1 ura3 his7 trp1* and TWY316, *MAT***a** *mec3-1 ura3 his3 trp1* were provided by T.Weinert (University of Arizona, Tucson). Strain PK110A-15, *MAT*α *esr1-1 leu2-1 his4 can1 ura3 cyh2 ade6 ade2* was provided by H.Ogawa (Osaka University). Genetic methods and yeast growth media were according to Rose *et al.* (1990).

Mutagenesis of the PRI1 gene

The procedure to generate two-codon insertions has already been described (Barany, 1988; Foiani *et al.*, 1994). Briefly, *pri1-M2* (T122-**DP**-C123), *pri1-M3* (R164-**RI**-R165), *pri1-M4* (N186-**GS**-V187), *pri1-M5* (N192-**GS**-V193), *pri1-T1 (*L229-**GS**-E230), *pri1-M6* (L297-**RI**-R298), *pri1-M7* (E397-**RI**-R398) and *pri1-H2* (E403-**DP**-P404) have been produced by using plasmid pFE139 and the TAB-linker 5′-CGGATC-3′. The *pri1-sM7* (E397-RA-R398) and *pri1-sH2* (E403-SS-P404) were generated in plasmid pFE5 by using the TAB-linker 5′-CGAGCT-3′. The two amino acid in-frame insertions obtained (bold letters, one letter code) within the *PRI1* open reading frame are indicated in parentheses next to each *pri1* allele, together with their positions in the p48 amino acid sequence. The pFE139 and pFE5 derivative plasmids carrying the two-codon insertions listed above were used to transform strains CY124 and YLAN, respectively, and transformants were tested at different temperatures for their ability to lose the wild-type *PRI1* copy carried, respectively, on the pFE5 and pFE139 plasmids.

Mapping the pri1-M4-dependent step within the cell cycle by reciprocal shift experiments

The rationale of reciprocal shifts to map the order of events during the yeast cell cycle has been described previously (Hereford and Hartwell, 1974; Hartwell, 1976). The conditions used to map the *pri1-M4*-dependent step were similar to those previously described to map the *pol12-T9*-dependent step (Foiani *et al.*, 1994). Briefly, α-factor pre-synchronized cells were arrested at 36°C for 2 h and then plated on YPD at the permissive temperature in the presence of 0.3 M HU. Alternatively, cells were held in 0.1 M HU for 2 h at the permissive temperature and then plated on YPD at 37°C in the absence of HU. The percentage of one large budded cell or of two adjacent budded cells after the second incubation on solid medium was scored microscopically (Foiani *et al.*, 1994). Since in both shifts $>$90% of the cells failed to divide (one large budded cell), we conclude that the *pri1-M4*-dependent step and the HU-dependent step are interdependent (Hereford and Hartwell, 1974; Hartwell, 1976).

Preparation of yeast extracts, Western blot analysis and immunoprecipitation procedures

The preparation of total protein extract from trichloroacetic acid (TCA)-treated cells and the procedure of Western blot analysis have been described already (Foiani *et al.*, 1994), as well as the preparation of non-denaturing protein extracts and immunoprecipitation of the pol α–primase complex (Ferrari *et al.*, 1996). Immunological reagents against the different subunits of the pol α–primase complex and against Rad53p have been also described (Foiani *et al.*, 1995; Sun *et al.*, 1996). To better resolve differences in electrophoretic mobility due to protein phosphorylation (Figure 7B), protein extracts were separated on 30 cm SDS–polyacrylamide gels containing 17% acrylamide and 0.072% bis-acrylamide at 150 V for 20 h.

Determination of DNA damage-induced cell cycle delay

To measure cell cycle delay at the G_1–S boundary in response to UV treatment, log-phase cultures were blocked in G_1 with 2 μg/ml of α-factor as previously described (Foiani *et al.*, 1994), plated in YPD and UV irradiated with 45 J/m^2. Cells were then washed from plates, rinsed to remove pheromone and resuspended in fresh YPD at 25°C. At timed intervals, samples were removed for FACS analysis, and ~400 cells were plated in triplicate onto YPD plates to measure cell survival after 3 days of incubation at 25°C. Cell cycle delay during S phase was analyzed by using the procedure described by Paulovich and Hartwell (1995). Briefly, α-factor pre-synchronized cells were released from the α-factor block in YPD medium containing 0.02% MMS. At timed intervals, samples were collected for FACS analyisis and measurement of cell survival, as described above. To analyze cell cycle delay at the G_2–M boundary in response to UV treatment, log-phase cultures were first blocked for 110 min in G_2/M by nocodazole (5 μg/ml) and dimethylsulfoxide (1%) and then plated on YPD and UV irradiated with 45 J/m^2. Cells were washed from the plates, rinsed to remove nocodazole, and resuspended in fresh YPD at 25°C. At timed intervals, cells were collected, and the percentage of uni- and bi-nucleate cells was scored microscopically after staining with 4′,6-diamidino-2-phenylindole (DAPI).

FACS analysis

Cells were grown in the appropriate media, sonicated for 15 s, collected by centrifugation and suspended in 70% ethanol for 16 h. Cells were then washed in 0.25 M Tris–HCl (pH 7.5), and suspended in the same buffer containing 2 mg/ml of RNase A. Samples were incubated for 12 h at 37°C, collected by centrifugation and the pellet was resuspended in 0.5 M pepsin freshly dissolved in 55 mM HCl. Cells were then washed in 180 mM Tris–HCl (pH 7.5), 190 mM NaCl, 70 mM $MgCl_2$ and stained in the same buffer containing 50 μg/ml of propidium iodide. Samples were then diluted 10-fold in 50 mM Tris–HCl (pH 7.8) and analyzed by using a Becton Dickinson FACScan.

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