Activation of Rad53 kinase in response to DNA damage and its effect in modulating phosphorylation of the lagging strand DNA polymerase

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The *Saccharomyces cerevisiae* Rad53 protein kinase is required for the execution of checkpoint arrest at multiple stages of the cell cycle. We found that Rad53 autophosphorylation activity depends on *in trans* phosphorylation mediated by Mec1 and does not require physical association with other proteins. Uncoupling *in trans* phosphorylation from autophosphorylation using a *rad53* kinase-defective mutant results in a dominant-negative checkpoint defect. Activation of Rad53 in response to DNA damage in G₁ requires the Rad9, Mec3, Ddc1, Rad17 and Rad24 checkpoint factors, while this dependence is greatly reduced in S phase cells. Furthermore, during recovery from checkpoint activation, Rad53 activity decreases through a process that does not require protein synthesis. We also found that Rad53 modulates the lagging strand replication apparatus by controlling phosphorylation of the DNA polymerase α -primase complex in response to intra-S DNA damage.

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Introduction

In response to genotoxic agents, cell cycle blocks or alterations of particular cellular structures, all eukaryotic cells activate a set of surveillance mechanisms, called checkpoints, (for reviews, see Carr and Hoekstra, 1995; Elledge, 1996; Paulovich *et al.*, 1997; Weinert, 1998). A subset of these mechanisms is represented by the DNA damage checkpoint, which is triggered by DNA lesions. The activation of this signal transduction pathway leads to a delay of cell cycle progression to prevent replication and segregation of damaged DNA molecules, and to induce transcription of several DNA repair genes (Paulovich *et al.*, 1997; Weinert, 1998). Increasing evidence indicates that defects in the cellular response to DNA damage are relevant for the early stages of carcinogenesis as they

cause increased mutagenesis and genomic instability (reviewed in Hartwell and Kastan, 1994; Weinert, 1997).

The mechanisms that control these responses have been studied in different organisms, including the yeast *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, which have been invaluable in providing the genetic tools to dissect the checkpoint response pathways.

In *S.cerevisiae*, the DNA damage checkpoint acts at three stages of the cell cycle: at the G₁/S transition, during S phase and at the G₂/M boundary, and the genetic requirements for proper DNA damage response in G₁, S and G₂ are at least partially distinct from each other (Paulovich *et al.*, 1997; Weinert, 1998). These differences may be linked to the recognition of the DNA lesions. In fact, it is possible that DNA damage is more accessible or can only be processed in certain periods of the cell cycle, and the options for the different types of repair processes might be different in G₁, S and G₂ cells.

Two essential genes, *MEC1* and *RAD53*, play a central role in the DNA damage checkpoint at all the cell cycle stages. Mec1 is a member of the evolutionarily conserved subfamily of phosphatidylinositol 3-kinase (PI3-kinase), which includes budding yeast Tel1, fission yeast Rad3, mammalian ATM and ATR and DNA-dependent protein kinase (DNA-PK) (Elledge, 1996). The similarity to DNA-PK, a protein kinase activated by binding to DNA and stimulated by the association of the Ku complex (Smith and Jackson, 1999), has suggested that Mec1 can also act as a protein kinase whose activity might be influenced by the association with other checkpoint proteins (Longhese *et al.*, 1998). However, the biochemical properties and protein–protein interactions of Mec1 remain speculative in the absence of any biochemical data on the *MEC1* gene product. Rad53 is an essential protein kinase required for cell cycle arrest in response to replication blocks and DNA damage (Allen *et al.*, 1994; Weinert *et al.*, 1994). Rad53 is phosphorylated in response to genotoxic treatments and this phosphorylation depends on the function of several DNA damage checkpoint genes (Sanchez *et al.*, 1996; Sun *et al.*, 1996). Rad53 has two FHA (forkhead-associated) domains, and Rad9, another checkpoint protein, interacts with the C-terminal FHA domain of Rad53 (Sun *et al.*, 1998), raising the possibility that Rad53 kinase activity might be influenced by association with other proteins.

Besides Rad9, other factors involved in the DNA damage response pathway include Mec3, Ddc1, Rad17 and Rad24. Rad17 exhibits a structural similarity to the proliferating cell nuclear antigen (PCNA) (Thelen *et al.*, 1999), while Rad24 is related to replication factor C (RF-C) (Lydall and Weinert, 1997), a protein complex that during DNA replication binds to template–primer junctions and loads PCNA onto the DNA, thereby recruiting replicative DNA polymerases (Waga and

Stillman, 1998). Even though the function of these checkpoint proteins is still unknown, it has been suggested that they might participate in DNA damage recognition and/or processing (Weinert, 1998).

Significant progress has been made in identifying many of the factors involved in the DNA damage checkpoint pathway, but very little is known about the physiological downstream targets. It has been suggested that the DNA replication machinery might be the final target of the DNA damage checkpoint response, which expands the length of S phase in the presence of genotoxic agents (Paulovich and Hartwell, 1995). Indeed, some replication factors have been implicated in the checkpoints, including DNA polymerase ϵ (Pol2), replication protein A (RP-A) and the DNA polymerase α -primase complex (pol-prim) (Navas *et al.*, 1995; Longhese *et al.*, 1996; Marini *et al.*, 1997). Pol2, whose role in DNA replication is still uncertain (Kesti *et al.*, 1999), is thought to be required for an early step in checkpoint activation, possibly as a sensor of DNA damage (Navas *et al.*, 1995). RP-A is involved in replication, recombination and repair (Wold, 1997) and, in response to DNA damage, is phosphorylated through a mechanism dependent upon Mec1, but not on Rad53 (Brush *et al.*, 1996). Moreover, the pol-prim complex, which is absolutely required for initiation of DNA synthesis at origins of replication and for lagging strand DNA synthesis (Foiani *et al.*, 1997), acts downstream of Rad53 and may represent one of the targets of the checkpoint pathway (Marini *et al.*, 1997).

It has been shown that checkpoint activation prevents the firing of late replication origins in the presence of genotoxic agents (Santocane and Diffley, 1998; Shiraige *et al.*, 1998), suggesting that either initiation at late origins or some other step of DNA synthesis is controlled by Mec1 and Rad53. Although the checkpoint targets that prevent late origin firing are still unknown, it has been suggested that Mec1 and Rad53 might modulate the activity of the Cdc28 and Cdc7 kinases that regulate initiation of DNA synthesis at the origins (Santocane and Diffley, 1998).

However, it is not clear yet whether the checkpoint negatively regulates S phase or rather promotes a specialized intrinsically slow replication process, or both. In fact, there are some analogies between the slow replication process occurring in the presence of genotoxic agents and the process that couples replication to recombination, which has been described in prokaryotes and in yeast (Malkova *et al.*, 1996; Kogoma, 1997; Foiani *et al.*, 1998). Intriguingly, all the replication factors implicated so far in the checkpoints are also involved in a repair pathway activated by a double strand break (DSB) and which couples replication to recombination (Holmes and Haber, 1999).

Here we have analysed the activity of the Rad53 protein kinase in response to genotoxic agents using an *in situ* autophosphorylation assay. Our data demonstrate that Rad53 activation does not require physical association with other proteins or nucleic acids and depends upon a functional Mec1. We show that activation of Rad53 requires the Rad9, Mec3, Ddc1, Rad17 and Rad24 checkpoint factors in G₁, while this dependency is strongly reduced when cells progress through S phase. Moreover, cells recovering from checkpoint activation inactivate

Rad53 through a process that does not require protein synthesis. Finally, we show that the lagging strand replication apparatus is controlled by Rad53 that prevents phosphorylation of the pol-prim complex in response to intra-S DNA damage.

Results

An *in situ* renaturation assay (ISA) to measure Rad53 autophosphorylation activity

To monitor Rad53 kinase activation directly and to begin to understand how Rad53 is able to transduce incoming signals, we have developed a renaturation assay to measure Rad53 autophosphorylation *in situ* (see Materials and methods for details).

As shown in Figure 1, when extracts prepared from wild-type exponentially growing cells were tested with the ISA, four major bands ranging in size from 45 to 60 kDa were detected and the intensity of these active kinase bands did not change when extracts were prepared from cells previously treated with the genotoxic agents hydroxyurea (HU) and methymethanesulfonate (MMS).

It has been shown that yeast cells respond to HU and MMS treatment by activating a signal transduction pathway that leads to Rad53 phosphorylation (Sanchez *et al.*, 1996; Sun *et al.*, 1996; and Figure 1). We found that an autophosphorylation activity with the same electrophoretic mobility as Rad53 was barely detectable in untreated wild-type extracts, but the intensity of this active band dramatically increased after HU and MMS treatment (Figure 1). Two control experiments indicate that the active kinase band indeed corresponds to Rad53: first, HU and MMS treatment of the kinase-defective *rad53-K227A* mutant strain caused phosphorylation of the Rad53 mutant protein without a concomitant increase in the autophosphorylation activity (Figure 1); and second the active Rad53 band was shifted to the expected higher molecular weight form when extracts were prepared from *rad53* mutant cells carrying a 9Myc-RAD53 gene on a plasmid (Figure 1). Both the Myc-tagged and the mutant Rad53 proteins were phosphorylated, but only the Myc-Rad53 protein was active in the ISA.

We failed to detect any fluctuation in the basal level of Rad53 autophosphorylation activity in untreated wild-type cycling cells synchronized by α -factor treatment (data not shown). We have also followed the kinetics of Rad53 activation in response to DNA damage. In wild-type cells released from a G₁ block in the presence of MMS, Rad53 underwent a significant mobility shift during the approximate time of S phase, which also correlated with the increase in Rad53 autophosphorylation activity (Figure 2A). When the same experiment was performed with the kinase-defective *rad53-K227A* mutant allele, Rad53 modification started 10 min earlier than in wild-type cells, but the autophosphorylation activity was barely detectable (Figure 2B). The anticipated modification timing can be related to the faster cell cycle progression observed by fluorescence activated cell sorting (FACS) of *rad53-K227A* cells under damaging conditions and is probably the result of *in trans* phosphorylation events. Wild-type cells released from a G₁ block in the presence of HU started to accumulate more slowly migrating Rad53 isoforms 30 min after the release, which correlates with

Table 1. Plasmids and strains used in this study

Plasmid/strain	Description/genotype	Reference/source
pNB187	YCp50 (<i>URA3</i> , <i>ARS1</i> , <i>CEN4</i>) with <i>GAL1</i> promoter	P.Novick
pNB187-rad53D339A	<i>rad53-D339A</i> coding sequences behind <i>GAL1</i> promoter in pNB187	D.Stern
pGALHA4CLB2	<i>HA4CLB2</i> coding sequences behind <i>GAL1</i> promoter in YCplac33 (<i>URA3</i> , <i>ARS1</i> , <i>CEN4</i>)	D.Baroni
pGALCLB5HA3	<i>CLB5HA3</i> coding sequences behind <i>GAL1</i> promoter in YIplac211 (<i>URA3</i>)	E.Schwob
pMHT	Δ <i>NSIC1myc</i> (stable Sic1) coding sequences behind <i>GAL1</i> promoter (<i>URA3</i>)	J.Diffley
pCH8	<i>rad53-K227A::KanMX4</i> in pRS316	this study
pCH10	<i>RAD53-9myc</i> in Ycplac111 (<i>CEN4</i> , <i>ARS1</i> , <i>LEU2</i>)	this study
pCH12	<i>rad53-D339A</i> coding sequences behind <i>GAL1</i> promoter in YIplac128 (<i>LEU2</i>)	this study
K699	<i>MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100</i>	K.Nasmyth
K700	<i>MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100</i>	K.Nasmyth
YLL134	<i>MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 mec3Δ::TRP1</i>	Longhese <i>et al.</i> (1997)
CY427	<i>MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 rad9Δ::URA3</i>	Marini <i>et al.</i> (1997)
CY387	<i>MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 pri1-M4</i>	Marini <i>et al.</i> (1997)
YLL9	<i>MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 pol1-1</i>	M.P.Longhese
K699-M2	<i>MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 rfa1-M2</i>	Longhese <i>et al.</i> (1996)
H2C2A1	<i>MATa ura1 his7 cdc2-2</i>	L.Hartwell
K4083	<i>MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 cdc4-2</i>	K.Nasmyth
CY1884	<i>MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 cdc28-13</i>	this study
H5CIB1	<i>MATa ura1 his7 cdc5-1</i>	L.Hartwell
H8CIA1	<i>MATa ura1 his7 cdc8-1</i>	L.Hartwell
H9CIB1	<i>MATa ura1 his7 cdc9-1</i>	L.Hartwell
DMP2537/5C	<i>MATa ade2-1 leu2-3,112 his3-11,15 ura3 can1-100 cdc7-1 HA2DDC1::LEU2::ddc1</i>	M.P.Longhese
CY2604	<i>MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 GAL1-rad53-D339A::LEU2 GAL1-ΔNSIC1myc::URA3</i>	this study
SS111-2-12	<i>MATa ade2-101 trp1-289 ura3-1,2 gal2 can1 pol2-12</i>	J.Campbell
SS111-2-11	<i>MATa ade2-101 trp1-289 ura3-1,2 gal2 can1 pol2-11</i>	J.Campbell
TC102-2-11	<i>MATa leu2 ura3-52 can1 pol2-11</i>	J.Campbell
TC102-2-12	<i>MATa leu2 ura3-52 can1 pol2-12</i>	J.Campbell
DMP2541/8A	<i>MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA2DDC1::LEU2::ddc1 mec1-1 sm11</i>	Paciotti <i>et al.</i> (1998)
CY2817	<i>MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 rad53K227A <pCH10RAD53-9myc LEU2></i>	this study
CY2321	<i>MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 GALCLB5HA3::URA3</i>	this study
CY1870	<i>MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 <pGALHA4CLB2 URA3></i>	this study
CY947	<i>MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 <pNB187 GAL1-rad53-D339A URA3></i>	this study
CY589	<i>MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 <pNB187 URA3></i>	Sun <i>et al.</i> (1996)
CY2034	<i>MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 KanMX4 rad53-K227A</i>	this study
YLL244	<i>MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 ddc1Δ::KanMX4</i>	Longhese <i>et al.</i> (1997)
DMP1913/20B	<i>MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 rad24Δ::TRP1</i>	M.P.Longhese
DMP1913/11C	<i>MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 rad17Δ::LEU2</i>	M.P.Longhese

Plasmids are indicated by parentheses.

Fig. 1. *In situ* autophosphorylation assay for Rad53 protein kinase. Log-phase (L) cultures of isogenic strains K699 (*RAD53*), CY2817 (*rad53* [*RAD53-MYC*]) or CY2034 (*rad53*) were treated for 3 h with 0.2 M HU (H) or 0.02% MMS (M). A 25 μ g aliquot of total protein was prepared from the indicated strains and analysed by ISA or Western blotting using anti-Rad53 antibodies as described in Materials and methods.

the increase in autophosphorylation activity (Figure 2C). Again, Rad53 phosphorylated isoforms were detectable in *rad53-K227A* mutant cells, but no autophosphorylation activity was measured by the ISA (Figure 2D).

When HU was removed from the medium, cells recov-

ered from the cell cycle block and restored a normal cell cycle progression. To test whether during recovery the cells adapt to the high level of Rad53 activity or, instead, turn Rad53 off, we treated wild-type cells with HU to induce checkpoint activation and then we removed it to allow recovery. As shown in Figure 2E, Rad53 activity began to decrease rapidly after HU removal and, concomitantly, cells started to proceed through S phase. However, it should be pointed out that even 3 h after the release from the HU block, when the cells were already in the next cell cycle, Rad53 activity was well above the basal level. Western blot analysis showed that the Rad53 phosphorylated isoforms progressively disappeared during recovery with the same kinetics observed for Rad53 activity with the ISA. These data indicate that recovery from HU correlates with a decrease in Rad53 activity. Moreover, residual Rad53 activity observed at late time points after the HU release is not sufficient to cause a cell cycle block, suggesting either that checkpoint activation requires a threshold of Rad53 activity or that the cell is able to adapt to a low level of Rad53 activity during recovery.

We then addressed whether the decrease of Rad53 activity observed during recovery was dependent upon

Fig. 2. Rad53 activity in response to DNA damage and during recovery. (A–D) Log-phase (Log) cultures of isogenic strains K699 (*RAD53*) (A and C) or CY2034 (*rad53*) (B and D) were pre-synchronized by α -factor treatment (α F) and released from the G₁ block in YPD with 0.02% MMS (A and B) or 0.2 M HU (C and D). Samples were taken at the time points indicated and analysed by FACS. A 25 μ g aliquot of total protein was prepared and analysed by ISA (Kin) or Western blotting. (E and F). Log-phase (Log) cultures of the *RAD53* strain were treated with 0.2 M HU for 3 h (HU), washed with the same volume of YPD to remove HU, and released from the HU block in YPD with (F) or without (E) 10 μ g/ml cycloheximide. Samples were taken at the indicated time points and analysed by FACS. A 25 μ g aliquot of total protein was prepared and analysed by ISA (Kin) or Western blotting.

protein synthesis. As shown in Figure 2F, wild-type cells released from the HU block in the presence of cycloheximide were able to proceed through S phase even though with slightly slower kinetics compared with the control cells, but failed to enter mitosis. Therefore, all the factors required to proceed through S phase during recovery have already been synthesized at the HU block, while the failure to execute mitosis in the presence of cycloheximide has already been described (Burke and Church, 1991). Since the decrease of Rad53 activity during recovery occurs with the same kinetics with or without cycloheximide treatment, the factor(s) required to turn Rad53 off seems to be already present at the HU block.

Altogether, the data above suggest that Rad53 is phosphorylated *in trans* in response to DNA damage and that this phosphorylation event activates an autophosphorylation reaction that can be measured by the ISA. The peculiarity of such an *in situ* renaturation assay also indicates that Rad53 autophosphorylation activity does not require structural association with other proteins, and that the very low Rad53 autophosphorylation activity observed under unperturbed conditions is not due to the presence of putative inhibitors. Moreover, our finding that Rad53 activity decreases during recovery suggests the existence of a cellular pathway required to turn Rad53 off, probably to allow cell cycle recovery after checkpoint activation.

Rad53 activity in different checkpoint- and cell cycle-defective mutants

The finding that phosphorylation of Rad53 caused by treatment with genotoxic agents is the cumulative result of *in trans* and autophosphorylation events prompted us to re-examine the genetic requirements of Rad53 activation by using the ISA.

We first tested the capacity of *cdc* mutants defective in the execution of critical cell cycle transition steps to activate Rad53 after shift to the restrictive temperature in the presence of HU or MMS (Figure 3A). The *cdc28-13* allele causes arrest at START at the restrictive temperature (Hereford and Hartwell, 1974) and, as shown in Figure 3A, in *cdc28-13*-arrested cells HU treatment failed to cause Rad53 activation, while fully active Rad53 was detectable in MMS-treated cells. The same result was obtained with *cdc4* mutant cells which arrest post-START in late G₁ (Schwob *et al.*, 1994). *cdc7-1* mutant cells fail to initiate DNA synthesis at the restrictive temperature (Hereford and Hartwell, 1974). MMS treatment in *cdc7-1* cells led to Rad53 activation, while, in the presence of HU, Rad53 activity was significantly lower. *POLI/CDC17*, *CDC2*, *CDC9* and *CDC8* respectively code for the catalytic subunits of DNA polymerase α , DNA polymerase δ , DNA ligase and thymidylate kinase (Murray and Hunt, 1993), and mutants in the corresponding genes arrest in S phase at the restrictive temperature. As shown in Figure 3A, in

Fig. 3. Rad53 activity in yeast strains defective in cell cycle progression and checkpoint response. (A) Log-phase cultures of strains K699 (wt), CY1884 (*cdc28*), K4083 (*cdc4*), DMP2537/5C (*cdc7*), YLL9 (*pol1*), H2C2A1 (*cdc2*), H9CIB1 (*cdc9*), H8CIA1 (*cdc8*) and H5C1B1 (*cdc5*) grown at 23°C were shifted to the restrictive temperature (37°C) for 4 h (0). The terminal phenotype of the *cdc* mutants was monitored microscopically. Then 0.2 M HU (H) or 0.02% MMS (M) was added for an additional 4 h at 37°C. A 25 µg aliquot of total protein was prepared and analysed by ISA (Kin) or Western blotting. (B) Log-phase cultures (L) of strains K699 (wt), CY2034 (*rad53*), DMP2541/8A (*mec1*), CY427 (*rad9*), DMP1913/11C (*rad17*), DMP1913/20B (*rad24*), YLL244 (*ddc1*), YLL134 (*mec3*), CY387 (*pri1*), K699-M2 (*rfa*) and SS111-2-11 (*pol2*) were treated with 0.2 M HU (H) or 0.02% MMS (M) for 3 h at 25°C. Cell cycle progression was analysed by FACS (data not shown). In the case of *pol2* mutants, the experiment was repeated at 30 and 37°C with analogous results also in strains TC102-2-11 and TC102-2-12 (data not shown). A 25 µg aliquot of total protein was prepared and analysed by ISA (Kin) or Western blotting. (C) Log-phase cultures of the same strains indicated in (B) were pre-synchronized in G₁ by adding 20 µg/ml of α-factor. Each culture was kept in G₁ for an additional hour with (+) or without (–) 2 µg/ml 4NQO. The incubations were carried out at 25°C. A 25 µg aliquot of total protein was prepared and analysed by ISA (Kin) or Western blotting.

response to either HU or MMS treatment, Rad53 was fully active in *pol1*, *cdc2*, *cdc9* and *cdc8* mutant cells at the restrictive temperature. Moreover, in these mutant backgrounds, Rad53 was partially active in the absence of any treatment probably due to defective DNA synthesis resulting in checkpoint activation in the absence of genotoxic agents. Finally, in *cdc5* mutant cells that arrest in M phase at the non-permissive temperature (Murray and Hunt, 1993), MMS but not HU treatment led to Rad53 activation.

In conclusion, this analysis indicates that MMS treatment leads to Rad53 activation at any stage of the cell cycle, while Rad53 activation in response to HU treatment appears to be S phase specific. However, it should be pointed out that Rad53 activation was very low when the MMS treatment was carried out at 25°C in wild-type G₁-arrested cells compared with cells experiencing S phase (data not shown), suggesting that the capacity of MMS to activate Rad53 is enhanced at 37°C and is maximal during S phase (Marini *et al.*, 1997; Vialard *et al.*, 1998).

Several checkpoint factors have been placed upstream of Rad53 in the pathway leading to Rad53 phosphorylation (Weinert, 1998), and mutations in the corresponding genes result in the inability to slow down cell cycle progression in response to HU or MMS treatment. As shown in

Figure 3B, Rad53 activity in response to HU or MMS treatment was greatly reduced in a *mec1-1* mutant background. Conversely, the Rad53 autophosphorylation reaction in response to HU treatment was unaffected in *rad9Δ*, *rad17Δ*, *rad24Δ*, *ddc1Δ* or *mec3Δ* cells, although a partial reduction in the activity was detectable in the same mutant cells after MMS treatment.

Other DNA replication proteins have also been implicated in the DNA damage checkpoint pathway, including DNA polymerase ε, pol-prim and RP-A (Navas *et al.*, 1995; Longhese *et al.*, 1996; Marini *et al.*, 1997). Figure 3B shows that in *pri1*, *rfa1* and *pol2* checkpoint-defective mutants, Rad53 could carry out the autophosphorylation reaction in response to HU and MMS treatment, although in MMS-treated *pol2* cells the Rad53 activity was lower than in wild-type cells. In *pri1*, *rfa1* and *pol2* mutant cells, Rad53 activity was slightly higher in untreated cells compared with wild-type cells, probably as a consequence of defective DNA replication.

These results indicate that, in response to HU treatment, Mec1 is absolutely required for Rad53 activation, while Rad9, Rad17, Rad24, Mec3 and Ddc1 appear to be dispensable. Conversely, Rad9, Rad17, Rad24, Mec3 and Ddc1 seem to be partially required for proper Rad53 activation in response to MMS treatment, which is again fully dependent on Mec1. Moreover, our finding that Rad53 is fully active in response to HU and MMS treatment in *pri1* and *rfa1* cells suggests that the checkpoint defect exhibited by these replication mutants is unlikely to be related to their inability to activate Rad53. Pol2 is thought to be required for checkpoint activation and Rad53 phosphorylation in response to HU treatment, and *pol2-12* mutants fail to delay cell cycle progression in the presence of HU (Navas *et al.*, 1995). It was somewhat surprising to find that in HU-treated *pol2-12* cells Rad53 was fully active and did not accumulate elongated mitotic spindles to a significant extent (data not shown). Since it has been suggested that *pol2* checkpoint defects are enhanced at higher temperatures (Navas *et al.*, 1996), we also tested the effect of the *pol2-11* and *pol2-12* mutations at 30 and 37°C. We found that Rad53 was fully active in HU-treated *pol2* mutant cells even at 37°C (data not shown) and, therefore, we conclude that, in our experimental conditions, the *pol2* checkpoint defect in the presence of HU is not due to a failure in activating Rad53. However, Rad53 activation was partially reduced in MMS-treated *pol2* cells.

G₁-arrested cells treated with a variety of genotoxic agents are able to delay entry into S phase by activating the Mec1- and Rad53-dependent checkpoint pathway. We therefore tested whether Rad53 activation in response to DNA damage in G₁ was affected in the mutant backgrounds analysed in Figure 3B. As shown in Figure 3C, we found that Rad53 was fully active in wild-type cells arrested in G₁ and treated with 4-nitro-quinoline-*N*-oxide (4NQO) throughout the G₁ block, while Rad53 activity was not detectable in treated *mec1*, *rad9*, *rad17*, *rad24*, *mec3* and *ddc1* cells. Conversely *pri1*, *rfa1* and *pol2* mutants were still able to activate Rad53 under the same experimental conditions. Analogous results were obtained using MMS instead of 4NQO (data not shown).

These data indicate that Rad53 activation in G₁-arrested cells strongly depends upon functional Mec1, Rad9, Rad17, Rad24, Mec3 and Ddc1.

Relationship between Rad53 activation and S phase progression

We have found recently that the pol-prim complex may be one of the final targets of the intra-S DNA damage checkpoint pathway mediated by Rad53 (Marini *et al.*, 1997). Accordingly, the data presented in Figure 3 seem to exclude a role for pol-prim and possibly other replication complexes upstream of Rad53. However, so far, there is no evidence to exclude that these replication proteins may represent a direct substrate of Rad53.

Pol-prim is a highly regulated enzyme that undergoes cell cycle-dependent phosphorylation and dephosphorylation (Foiani *et al.*, 1997). The B subunit of the complex is phosphorylated early in S phase and dephosphorylated while cells are exiting from mitosis (Foiani *et al.*, 1995), and recent evidence indicates that its phosphorylation is dependent upon a functional Clb-Cdc28 complex (Desdouets *et al.*, 1998; G. Liberi and M. Foiani, unpublished data). In order to understand how pol-prim (and possibly the whole replication machinery) is regulated by the Rad53-dependent pathway in response to DNA damage, we have analysed the phosphorylation state of the pol-prim B subunit in wild-type cells released from a G₁ block in the presence of HU or MMS.

As shown in Figure 4A and B, B subunit phosphorylation, which usually occurs 20–30 min after release from α -factor (data not shown; Foiani *et al.*, 1995), was greatly delayed in the presence of MMS or HU, and this delay was reduced in a *rad53* mutant background.

We found that overexpression of the *rad53-D339A* kinase-defective allele under the control of a *GAL1*-inducible promoter causes a dominant checkpoint-defective phenotype. In fact, when cells were allowed to enter S phase in the presence of MMS to activate the checkpoint and in the presence of galactose to induce the expression of the *rad53-D339A* allele, cells progressed through S phase more quickly than control cells grown in MMS and raffinose (Figure 4C). Progression through the cell cycle was accompanied by a concomitant decrease in Rad53 activity (Figure 4C). An analogous reduction of the kinase activity was observed when the *rad53* kinase-defective allele was overexpressed in the presence of HU, although S phase progression was prevented by the drug (Figure 4D). Overexpression of a Rad53 kinase-defective form can determine a dominant checkpoint defect by assuming that the mutant protein is able to compete with the signals generated by DNA damage, thus leading to a reduction in the amount of active Rad53 capable of productively transducing the signal downstream. The results shown in Figure 4C also indicate that Rad53 kinase activity is required not only for checkpoint activation, but also for its maintenance throughout the treatment.

Overexpression of the *rad53* kinase mutant led to derepression of B subunit phosphorylation (Figure 4C and D) as was similarly observed in *rad53* mutant cells in the presence of HU and MMS (Figure 4B). Since the derepression of B subunit phosphorylation in a checkpoint-defective background was also observed in the presence of the S phase inhibitor HU, it is unlikely to be related to a cell cycle effect (Figure 4B and D). Altogether, our data suggest that Rad53 controls B subunit phosphorylation in response to genotoxic treatments.

We hypothesized that checkpoint activation either modi-

fies the replication machinery in such a way that the pol-prim B subunit is no longer available for phosphorylation, or that Rad53 negatively regulates the kinase responsible for B subunit phosphorylation (or modulates its substrate specificity).

Since phosphorylation of the B subunit is dependent upon its association with the DNA polymerase α catalytic subunit (Ferrari *et al.*, 1996), we tested whether pol-prim complex formation was influenced by HU or MMS treatment. By co-immunoprecipitation experiments, we found that pol-prim complex formation was not affected by DNA-damaging conditions (data not shown).

We then tested the possibility that the lack of B subunit phosphorylation in response to checkpoint activation could be brought about by negative regulation of the Clb-Cdc28 complex. Cells were first treated with HU to activate the checkpoint and then the kinase-defective *rad53* allele was overexpressed together with a stable version of the Clb-Cdc28 inhibitor Sic1 (Desdouets *et al.*, 1998). As shown in Figure 5A, inhibition of Clb-Cdc28 caused by *SIC1* overexpression prevented B subunit phosphorylation despite the concomitant production of a dominant-negative Rad53 form that normally leads to derepression of B subunit phosphorylation (see Figure 4D). The ISA performed on the same samples showed that Sic1 overexpression does not prevent the reduction in Rad53 activity caused by the expression of the kinase-defective *rad53* allele (data not shown). Similar results were obtained when cells were treated with MMS instead of HU in an otherwise identical experiment (data not shown).

These results indicate that B subunit phosphorylation requires a functional Clb-Cdc28 active complex even in a checkpoint-defective context. Moreover, these findings might suggest that Rad53 negatively regulates the Clb-Cdc28 complex in response to DNA damage. If this assumption is correct, it is expected that ectopic activation of Clb-Cdc28 under DNA-damaging conditions would lead to premature B subunit phosphorylation and possibly to checkpoint defects. As shown in Figure 5, when Clb5 or Clb2 were overexpressed in cells released from a G₁ block in the presence of MMS, cells were still able properly to delay both S phase progression, as indicated by FACS analysis (Figure 5C), and nuclear division as monitored microscopically (data not shown). However, Cdc28 ectopic activation led to premature B subunit phosphorylation after both HU (Figure 5B) and MMS treatment (data not shown). These data suggest that: (i) the B subunit is available for phosphorylation under DNA-damaging conditions; (ii) Clb-Cdc28 is either transiently inhibited or phosphorylates different substrates in response to checkpoint activation; and (iii) ectopic activation of Clb-Cdc28 is not sufficient to accelerate S phase progression in the presence of MMS.

It is somewhat surprising that mutations affecting the function of the replication machinery, such as the *prl1-M4* and *rfa1-M2* mutations, override the checkpoint in response to DNA damage (Longhese *et al.*, 1996; Marini *et al.*, 1997), while Cdc28 ectopic activation does not accelerate cell cycle progression under the same conditions. It is possible that the checkpoint pathway diverges downstream of Rad53 and that Clb-Cdc28 represents just one of the putative targets which has to be negatively

Fig. 4. Rad53 delays pol-prim B subunit phosphorylation in response to DNA damage. (A and B) Log-phase cultures of isogenic strains K699 (*RAD53*) or CY2034 (*rad53*) were pre-synchronized by α -factor treatment (α F) and released from the G_1 block in YPD containing 0.02% MMS or 0.2 M HU. Samples were taken at the times points indicated and 25 μ g of total protein were prepared and analysed by Western blotting using antibodies against the B subunit. The slower migrating form represents phosphorylated B subunit. Cell cycle progression was analysed by FACS (data not shown). (C) A log-phase culture (Log) of strain CY947, carrying *GAL1-rad53-D339A* on a plasmid, was grown in 2% raffinose and treated for 3 h with 0.02% MMS (0). Galactose (2%) was then added to half of the culture to overexpress *rad53*, while the other half was maintained in raffinose. Samples were taken at the times indicated and analysed by FACS. A 25 μ g aliquot of total protein was prepared and analysed by ISA to monitor Rad53 activity (Kin) or by Western blotting using anti-B subunit antibodies. (D) A log-phase culture of strain CY947 was grown in 2% raffinose and treated for 3 h with 0.2 M HU (0). Galactose (2%) was then added to half of the culture, while the other half was maintained in raffinose. Samples were taken at the times indicated and analysed by FACS (black histograms). The white histograms at the back represent the cell cycle distribution of the asynchronous cultures before α -factor treatment. A 25 μ g aliquot of total protein was prepared and analysed by ISA to monitor Rad53 activity (Kin) or by Western blotting using anti-B subunit antibodies.

regulated in order to slow down DNA replication in response to DNA damage.

Discussion

The DNA replication process *per se* is a source of intrinsic DNA damage as a result of stochastically occurring errors during nucleotide incorporation, and further complications may arise when cells are exposed to genotoxic agents while traversing S phase (Foiani *et al.*, 1998). In fact, the fragility of unwound replicating DNA exposed to extrinsic damage greatly exacerbates genetic instability in countless ways. Since the maintenance of genome integrity during cell division is of high priority for all living organisms, cells have to coordinate DNA replication with the checkpoints activated by DNA damage (Carr and Hoekstra, 1995; Elledge, 1996; Paulovich *et al.*, 1997; Weinert, 1998).

In the yeast *S.cerevisiae*, the *RAD53* gene product is known to play a central role in the S phase DNA damage checkpoint, which expands the length of S phase in the presence of genotoxic agents (Paulovich and Hartwell,

1995). Although there is evidence to indicate that Rad53 is phosphorylated in a Mec1-dependent manner as a consequence of DNA damage (Sanchez *et al.*, 1996; Sun *et al.*, 1996), it is still unclear how Rad53 is activated in response to incoming damage signals and how the signals are transduced to modulate DNA replication and cell cycle progression.

Here we describe a novel *in situ* autophosphorylation assay that represents a powerful tool to study the mechanisms leading to Rad53 activation. Previous assays to monitor Rad53 kinase activity *in vitro* relied on immunoprecipitation (IP) experiments and on substrates that have not been proven to correspond to physiological targets of the Rad53 kinase. Moreover, proteolysis and dephosphorylation artefacts cannot be excluded from IP experiments and there is always the possibility that immunologically unrelated kinases will be pulled-down as contaminants, therefore interfering with the assay itself. The trichloroacetic acid (TCA) treatment which we use in the ISA not only minimizes protein degradation and dephosphorylation by inactivating proteases and phosphatases, but also prevents artefactual Rad53 activation, which may be induced

Fig. 5. Rad53 prevents B subunit phosphorylation by counteracting Cdk1 activity. (A) The experiment was carried out as in Figure 4D. A log-phase culture of strain CY2604 carrying *GAL1-rad53-D339A* and *GAL1-SIC1* was grown in 2% raffinose and treated for 3 h with 0.2 M HU (0). Galactose (2%) was then added to half of the culture to overexpress both *rad53* and *SIC1*, while the other half was maintained in raffinose. Samples were taken at the times indicated and analysed by FACS. A 25 μ g aliquot of total protein was prepared and analysed by ISA to monitor Rad53 activity (data not shown) or by Western blotting using anti-B subunit antibodies. (B) Log-phase cultures of strains CY589 (vector), CY2321 (*GAL1-CLB5*) or CY1870 (*GAL1-CLB2*) were grown in 2% raffinose and treated for 3 h with 0.2 M HU (0). Galactose (2%) was then added to the cultures and samples were taken at the times indicated and analysed by FACS. A 25 μ g aliquot of total protein was prepared and analysed by Western blotting using anti-B subunit antibodies. (C) Log-phase cultures of the same strains shown in (B) were treated with 0.02% MMS for 3 h in 2% raffinose (0). Galactose (2%) was then added to the cultures and samples were taken at the time points indicated and analysed by FACS and microscopically to monitor nuclear division (data not shown). Samples were taken at the indicated times and analysed by FACS (black histograms). The white histograms at the back represent the cell cycle distribution of the asynchronous cultures before α -factor treatment.

by DNA fragmentation occurring during protein extraction. Finally, the ISA is performed *in situ*, making it very unlikely that the association with other proteins or nucleic acids will positively or negatively influence Rad53 kinase activity.

Rad53 is poorly autophosphorylated under unperturbed conditions but becomes heavily autophosphorylated when cells are treated with either HU or MMS. Our data also support the notion that damage-induced autophosphorylation is promoted by *in trans* phosphorylation mediated by other kinases, possibly by Mec1. Although the molecular mechanisms leading to Rad53 activation remain to be defined, it is possible that the Mec1-dependent phosphorylation modifies the catalytic properties of Rad53 kinase allowing its activation and autophosphorylation.

We have been able to uncouple *in trans* phosphorylation of Rad53 from autophosphorylation genetically by using the kinase-defective *rad53-D339A* mutant allele. The Rad53 mutant protein can still be phosphorylated *in trans* in response to DNA damage, but its overexpression causes a reduction in the level of wild-type Rad53 activation, strongly suggesting that the dominant-negative checkpoint defect exhibited by the *rad53-D339A* mutant is due to its inability to transduce incoming signals, thus further strengthening the analogy between Rad53 and other protein kinases involved in signal transduction pathways. We also

found that a functional checkpoint cannot be maintained when the dominant-negative *rad53* allele is overexpressed in DNA-damaging condition, despite the fact that the level of Rad53 activity is higher than in untreated cells. This finding suggests that a functional checkpoint probably requires a threshold level of Rad53 kinase and that Rad53 activity is required for checkpoint maintenance and not only for its activation.

Recovery from checkpoint activation correlates with Rad53 inactivation. This observation, together with the finding that *RAD53*-overexpressing cells, which exhibit a dramatic increase in the level of Rad53 activity, irreversibly arrest the cell cycle in response to DNA damage (M.Lopes and M.Foiani, in preparation), suggest that Rad53 inactivation is required for proper recovery from a cell cycle block. Given that the inactivating factors have already been synthesized under damaging conditions, it will be of interest to understand which are the mechanisms leading to Rad53 inactivation during recovery and those preventing Rad53 inactivation in the presence of DNA damage. Therefore, the cellular response mediated by Rad53 is likely to involve at least three steps: (i) *in trans* phosphorylation which is related to Rad53 activation; (ii) prevention of Rad53 inactivation in the presence of DNA damage signals; and (iii) Rad53 inactivation through dephosphorylation or specific degradation of phosphoryl-

ated Rad53. Impairment of the first two steps will cause a checkpoint defect, while a failure to inactivate Rad53 will lead to irreversible cell cycle arrest.

It has been shown recently that replication fork arrest leads to the formation of DNA strand breaks (Seigneur *et al.*, 1998). Moreover, HU treatment induces recombination (Galli and Schiestl, 1996), and cell viability in the presence of sub-lethal HU concentrations requires a functional DSB repair system, since cell survival is strongly reduced in a *rad52* genetic background (our unpublished observations). Hence, it is possible that HU treatment, besides blocking DNA replication by inhibiting ribonucleotide reductase, also causes the accumulation of DNA breaks. DNA strand interruptions might arise from the accumulation of early replication intermediates at origins of replication, since the formation of RNA primers is probably not prevented by dNTP depletion caused by the HU treatment. Similarly, MMS treatment, either directly or as a result of the repair process, might accumulate DNA strand breaks. Moreover, replication of alkylated DNA templates can cause the accumulation of strand interruptions due to re-initiation events downstream of the lesions.

From this perspective, it is evident that one of the main problem that needs to be addressed is the definition of the signals leading to DNA damage response and, in particular, to checkpoint activation. If the signal is represented by the primary damage, it must be assumed that highly specialized sensors have to be employed by the cell to recognize different types of lesions. Alternatively, it is conceivable that various types of damage may be converted to a common DNA structure (e.g. DNA breaks or accumulation of single-stranded DNA), which would represent the unique signal(s) for checkpoint activation (Nelson and Kastan, 1994; Foiani *et al.*, 1998). In this last scenario, replication of a damaged template might have an active role in generating strand interruptions that activate the checkpoint (Foiani *et al.*, 1998).

A key question is whether DNA replication under unperturbed conditions generates checkpoint signals. This assumption is supported by the observation that under normal conditions, both RP-A and Ddc1 are phosphorylated (Din *et al.*, 1990; Brush *et al.*, 1996; Paciotti *et al.*, 1998). When we analysed the phosphorylation state of Rad53 in a normal cell cycle, both by Western blotting and by the ISA, we failed to detect any fluctuations, suggesting either that Rad53 is not active during an unperturbed S phase or that Rad53 is activated in response to intrinsic DNA damage but our assays are not sensitive enough to detect such events.

We have analysed a set of checkpoint-defective mutants for their ability to activate Rad53 in response to HU or MMS treatment and, based on our results, three different classes of genes can be identified. The first class is represented by *MEC1*, which is required for proper Rad53 activation in the presence of HU or MMS. This result confirms previous observations (Sanchez *et al.*, 1996; Sun *et al.*, 1996) suggesting that Mec1, either directly or indirectly, is required for *in trans* phosphorylation of Rad53.

The extent of the Rad53 autophosphorylation reaction in HU-treated cells is not affected by mutations in a second class of genes which includes *rad17*, *rad24*, *mec3*, *ddc1* and *rad9*. The same mutations cause a partial

reduction in Rad53 autophosphorylation when cells are treated chronically with MMS during DNA replication, while they completely prevent Rad53 activation when the cells experience DNA damage in G₁ both by 4NQO and MMS treatment. It is possible that the function of the *RAD9*, *RAD24*, *RAD17*, *MEC3* and *DDC1* gene products is to monitor the primary damage caused by various genotoxic agents, and it may be expected that certain types of damage are recognized preferentially while others escape detection (as in the case of HU). Alternatively, the role of the *RAD9*, *RAD24*, *RAD17*, *MEC3* and *DDC1* gene products, either alone or together with other as yet unidentified repair and/or recombination factors, may be to recognize distortions in the double helix caused by different genotoxic agents and to generate a common intermediate (i.e. DNA breaks or single-stranded DNA) which would then be the signal for Mec1 activation.

The finding that Rad24, Rad17, Mec3, Ddc1 and Rad9 are only partially required to activate Rad53 when cells are treated with MMS during S phase may be related to the hypothesis that DNA replication in the presence of genotoxic agents generates DNA strand interruptions that are sufficient to activate the checkpoint (Foiani *et al.*, 1998). However, active processing of the damage to produce the appropriate signal would be necessary in G₁, explaining the dependency of Rad53 activation on Rad24, Rad17, Mec3, Ddc1 and Rad9 at this cell cycle stage. It must also be considered that damage recognition is likely to be affected by the chromatin structure which, in turn, is influenced by the cell cycle stage. DNA lesions can be recognized more easily in S phase than in G₁, and it is possible that specialized factors are required to unmask DNA damage in a G₁ chromatin context.

Our finding that in HU-treated *pol2* mutant cells Rad53 is fully active is in apparent contrast to previous observations suggesting a role for Pol2 as a sensor, upstream of Rad53 (Navas *et al.*, 1995). This discrepancy may be ascribed to the fact that in our experiments we used 0.2 M HU (instead of 0.15 M), which causes checkpoint activation but also strongly prevents DNA synthesis. In MMS-treated *pol2* cells, Rad53 activation is partially reduced. A possible explanation is that *pol2* cells are unable to replicate a damaged template properly and consequently fail to accumulate certain DNA intermediates which cause checkpoint activation. Interestingly, while the role of Pol ϵ in normal DNA replication is still unclear (Kesti *et al.*, 1999), recent observations have implicated this enzyme in a specialized replication process required to repair DSBs (Holmes and Haber, 1999).

We have shown that cells can initiate DNA replication in the presence of MMS and still become checkpoint defective following the expression of a dominant-negative form of Rad53, suggesting that the checkpoint modulates late origin firing and/or some late step of DNA replication. Accordingly, it has been found that in response to HU or MMS treatment, DNA replication initiates at early origins, while late origin firing is prevented in a Rad53-dependent manner (Santocanale and Diffley, 1998; Shiraige *et al.*, 1998).

It has been suggested that the replication machinery itself may represent one of the targets of the checkpoint pathway (Paulovich and Harwell, 1995) and, indeed, certain mutations in DNA replication genes cause a

checkpoint-defective phenotype. A third class of factors, such as RP-A and pol-prim, does not seem to play any role in the process leading to Rad53 activation in response to DNA damage, although mutations in the *RFA1* and *PR11* genes cause a checkpoint-defective phenotype (Longhese *et al.*, 1996; Marini *et al.*, 1997). The single-stranded DNA-binding protein RP-A is a three subunit complex whose function is essential in various aspects of DNA metabolism, including DNA replication, repair, recombination and transcription (Wold, 1997). It has also been shown that RP-A physically interacts with pol-prim during the replication process (Dorneiter *et al.*, 1992) and RP-A is hyperphosphorylated in response to DNA damage through a mechanism which is *MEC1*-dependent and *RAD53*-independent (Brush *et al.*, 1996). These data, together with our observation that in the *rfa1-M2* mutant Rad53 is fully activated in response to DNA damage, would genetically position RP-A downstream of Mec1 in a pathway diverging from that leading to Rad53 activation. We recently have suggested that DNA primase may be one of the final targets of the Rad53-mediated checkpoint response, and the finding that Rad53 is fully activated in a *pri1-M4* background further supports this conclusion. However, pol-prim does not seem to be a direct substrate of Rad53 and, therefore, other factors might mediate the transduction cascade from Rad53 to pol-prim.

The experiments described here indicate that phosphorylation of the pol-prim B subunit is controlled by Rad53 in response to HU and MMS treatment through a mechanism which probably involves the modulation of Clb-Cdc28 activity. Although the function of the B subunit is still unknown, it seems to be required specifically for the initial step of DNA synthesis and is phosphorylated periodically through mechanisms dependent upon the activity of CDK complexes (Foiani *et al.*, 1997). Furthermore, it has been shown that B subunit dephosphorylation correlates with pol-prim chromatin association, which is prevented by the activity of Clb-Cdc28 kinase (Desdouets *et al.*, 1998). The finding that B subunit phosphorylation is delayed under damaging conditions may suggest that the Clb-Cdc28 kinases are transiently inhibited in response to checkpoint activation.

Accumulating evidence from our laboratory indicates that B subunit phosphorylation is a marker of Clb-Cdc28 activity (G. Liberi and M. Foiani, unpublished observations). Therefore, the finding that this modification is inhibited by checkpoint activation suggested to us the possibility that Clb-Cdc28 may be the main modulator of the checkpoint response. However, Clb overexpression under DNA-damaging conditions can drive B subunit phosphorylation without causing faster cell cycle progression. It is likely that the checkpoint response is not a simple linear pathway leading to Clb-Cdc28 modulation and, consequently, to S phase delay. Other factors regulating S phase entry and progression may be involved, and accumulating evidence from several laboratories suggests a possible involvement of the Cdc7/Dbf4 protein kinase (Cheng *et al.*, 1999; Dohrmann *et al.*, 1999; Figure 6A).

Thus far, we have been trying to account for the S phase delay observed in response to DNA damage as a result of a negative regulation of the replication machinery. In an alternative scenario, the DNA damage response pathway might instead promote a specialized replication mechanism

Fig. 6. Model for the S phase DNA damage checkpoint. **(A)** A schematic representation of the checkpoint pathway which specifically delays progression through S phase in response to intra-S DNA damage is presented. Dashed lines indicate that other gene products not shown in the figure might mediate the indicated steps. Rad53 activation depends upon Mec1 which is able to sense DNA damage induced by HU and MMS treatments. In the case of MMS, other factors not shown in the figure (see Discussion for details) might contribute to the accumulation of DNA structures which are recognized by Mec1. Rad53 might modulate the activity of Cdk1 (as indicated by the asterisk) which is no longer able to phosphorylate pol-prim properly. Other possible targets of Rad53 could be represented by Cdc7. Mec1 is also involved in RP-A phosphorylation. **(B)** Protein phosphorylation is indicated in grey and black. During an unperturbed S phase, pol-prim becomes phosphorylated (black) while RP-A is hypophosphorylated (grey). Checkpoint activation in response to intra-S DNA damage prevents pol-prim phosphorylation while RP-A becomes hyperphosphorylated (black).

which is intrinsically slow. We have proposed recently that checkpoint activation might inhibit origin-dependent DNA synthesis, while promoting an alternative mode of DNA replication where new replication forks are assembled at DNA strand breaks and fork movement occurs via branch migration (Foiani *et al.*, 1998). This replication by a recombination mechanism has been described recently in *S.cerevisiae* cells, which are able to repair DSBs by using a specialized pathway which is dependent upon the lagging strand replication apparatus (Malkova *et al.*, 1996; Holmes and Haber, 1999).

If an alternative mode of DNA replication is promoted by checkpoint activation, the primary effect of CDK regulation would be to make available the lagging strand replication apparatus for this alternative replication process, rather than negatively to regulate S phase progression. However, our data indicate that the CDK regulation is not sufficient to promote a proper checkpoint response. RP-A, whose phosphorylation as a consequence of DNA damage is Mec1-dependent, might also play an important role in

promoting this alternative mode of replication, since RP-A is required for both recombination and replication and physically interacts with pol-prim (Figure 6B). This model might explain the finding that CDK ectopic activation does not override the checkpoint, while certain *rfal* and *pril* mutations cause a checkpoint-defective phenotype. Moreover, this replication-coupled recombination process is likely to be mechanistically more complex and slower than normal DNA synthesis, thus accounting for the expanded S phase observed in MMS-treated cells.

Materials and methods

Plasmids

Plasmid pCH8 was constructed by inserting the *KanMX4* cassette downstream of the *rad53-K227A* coding region into the blunted *BlpI* site of plasmid pRS316-*spk1-K227A* (Sun *et al.*, 1996). Plasmid pCH10 was constructed by cloning the *EcoRI-EcoRI RAD53* genomic fragment from plasmid pRS316-*RAD53* (Sun *et al.*, 1996) into plasmid Ycplac111 (Geits and Sugino, 1988). A DNA fragment encoding a 9Myc epitope was then inserted into the *NotI* site, which was created by PCR just before the stop codon of *RAD53*. Plasmid pCH12 was originated by cloning the *EcoRI-EcoRI* fragment from plasmid pNB187-*rad53-D339A* (Sun *et al.*, 1996) carrying *GAL1-rad53-D339A* into the *EcoRI* site of plasmid Ylplac128 (Gietz and Sugino 1988).

Yeast strains

Strain K1393 (*MAT α trp1 ade1 leu2 his2 cdc28-13*) kindly provided by K.Nasmyth, was backcrossed to strain K699 to produce strain CY1884. Strain CY2604, a K699 derivative, was produced by integrating the *BstXI*-linearized pCH12 plasmid at the *LEU2* locus and the *ApaI*-linearized pMHT plasmid at the *URA3* locus. Strain CY2321 is a K699 derivative and was produced by integrating the *EcoRV*-linearized pGLACLB5HA3 plasmid at the *URA3* locus. Strain CY2034 is a K699 derivative and was originated by integrating the *EcoRI*-linearized pCH8 plasmid at the *RAD53* locus.

In situ autophosphorylation assay

Yeast protein extracts were prepared from TCA-treated cells (see below). Equal amounts of proteins (25 μ g/sample), mixed with 1 $\times$ Laemmli buffer, were loaded onto 10% SDS-polyacrylamide gels and run according to standard procedures. After electrophoresis, gels were blotted onto PVDF (Immobilon-P, Millipore). The membranes were then subjected to a denaturation/renaturation protocol according to the procedure described by Ferrel and Martin (1991) with the following modifications. The denaturing step was for 1 h at room temperature in 7 M guanidine-HCl, 50 mM dithiothreitol (DTT; freshly prepared), 2 mM EDTA, 50 mM Tris-HCl pH 8.0. The membranes were then washed twice in 1 $\times$ Tris-buffered saline (TBS) buffer for 10 min at room temperature. Renaturation was carried out at 4°C for 12–18 h with weak shaking in 2 mM DTT (freshly prepared), 2 mM EDTA, 0.04% Tween-20, 10 mM Tris-HCl pH 7.5, 140 mM NaCl, 1% bovine serum albumin (BSA). The buffer was changed at least four times. The membranes were then washed for 60 min in 30 mM Tris-HCl pH 7.5 and equilibrated in kinase buffer (1 mM DTT, 0.1 mM EGTA, 20 mM MgCl₂, 20 mM MnCl₂, 40 mM HEPES-NaOH pH 8.0, 100 μ M sodium orthovanadate) for 30 min at room temperature. The autophosphorylation reaction was performed by incubating the membranes in kinase buffer in the presence of 10 μ Ci/ml of [γ -³²P]ATP for 1 h at room temperature. Membranes were then washed as follows: twice for 10 min in 30 mM Tris-HCl pH 7.5, once for 10 min in 30 mM Tris-HCl pH 7.5, 0.1% NP-40 (freshly prepared), 10 min in 30 mM Tris-HCl pH 7.5, 10 min in 1 M KOH, 10 min in water, 10 min in 10% TCA and 10 min in water. The filters were then dried and exposed.

Western blot analysis and immunological reagents

Preparation of yeast protein extracts from TCA-treated cells was performed as described in Foiani *et al.* (1999). The procedure for Western blot analysis and the antibodies against the B subunit have already been described (Foiani *et al.*, 1995). Antibodies against Rad53 were a gift from C.Santocanale and J.Diffley.

FACS analysis

FACS analysis was performed as described in Foiani *et al.* (1999).

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