A domain of Rad9 specifically required for activation of Chk1 in budding yeast

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Summary

The Rad9 protein is a key adaptor protein in Saccharomyces cerevisiae DNA damage checkpoint pathways. Its adaptor function is to link the activity of the Mec1 kinase to the activation of two parallel signalling pathways dependent on the Rad53 and Chk1 kinases. The mechanisms by which Rad9 interacts with, and activates, Rad53 are well understood. However, little was known about how Rad9 facilitates the activation of Chk1. We show here that the N-terminus of Rad9 is specifically important for phosphorylation and activation of the Chk1 kinase but not for the phosphorylation and activation of the Rad53 kinase. The Chk1 activation domain (CAD) of Rad9 is specifically important for signalling cell-cycle arrest after cdc13-1- and yku70Δ-induced telomere damage but not for tolerating ultraviolet-induced damage or inhibiting nuclease activity at telomeres. This work extends data showing that separable domains within the Rad9 adaptor protein allow it to activate two distinct kinase signalling pathways independently of each other.

Key words: RAD9, CHK1, Checkpoint, Yeast, Adaptor

Introduction

DNA damage checkpoints are mechanisms utilized by eukaryotic cells to monitor the integrity of their genomes and they play an important role in maintaining genomic stability. Checkpoints are signalling pathways built around the regulated activity of kinases. The kinases at the heart of DNA damage checkpoint signalling pathways are conserved from yeast to mammals, as are the accessory proteins that localize to DNA lesions and that regulate the activation of the kinases (Melo and Toczyski, 2002; Nyberg et al., 2002; Rouse and Jackson, 2002a).

In budding yeast, the major kinase involved in DNA damage checkpoint pathways is encoded by the essential gene MECl. Mec1 is a phosphoinositide 3-kinase-like kinase (PIKK), and its homologues in fission yeast and vertebrates are Rad3 and ATR (for ‘ATM and Rad3-related’), respectively (reviewed by Abraham, 2001; Nyberg et al., 2002). Two protein kinases, Rad53 and Chk1, act downstream of Mec1 in distinct pathways (Gardner et al., 1999; Sanchez et al., 1999). Rad53 and Chk1 are also conserved in fission yeast as Cds1 and Chk1, and in vertebrates as Chk2 and Chk1, respectively (Abraham, 2001; Rhind and Russell, 2000). Mec1 appears to be activated in virtually all pathways of DNA damage checkpoint signalling. Rad53 is activated following DNA damage and replication block (Allen et al., 1994; Navas et al., 1996; Sanchez et al., 1996; Weinert et al., 1994), whereas Chk1 is only required for arrest following a subset of DNA damage lesions and does not appear to be required following replication block (Liu et al., 2000; Sanchez et al., 1999). In Schizosaccharomyces pombe, there is a clearer distinction in function: Cds1 (Rad53) is activated following replication block (Lindsay et al., 1998) and Chk1 is activated following DNA damage (Walworth et al., 1993).

RAD9 was the first checkpoint gene to be defined (Weinert and Hartwell, 1988) but its biochemical function has only recently become apparent. Current models are that Rad9 is an ‘adaptor’ checkpoint protein whose function is to couple the activation of upstream kinases (Mec1) with downstream effector kinases (Rad53 and Chk1) (Melo and Toczyski, 2002). The role of adaptor proteins such as Rad9 seems to be both in ensuring specificity in checkpoint activation and in amplification of the damage signal (Gilbert et al., 2001). Other examples of adaptor or mediator proteins include Mrc1 (yeast), Crb2 (S. pombe), Claspin (Xenopus) and BRCA1 (human) (Melo and Toczyski, 2002) and a new mammalian mediator protein, MDC1, has recently been described (Goldberg et al., 2003; Lou et al., 2003a; Lou et al., 2003b; Stewart et al., 2003).

In both yeast and human cells, at least two checkpoint protein complexes appear to be recruited to sites of DNA lesions. One complex contains a PIKK kinase and its interacting partner (Mec1 and Ddc2 in budding yeast), whereas a second hetero-trimeric complex is recruited independently (Mec3-Rad17-Ddc1 in budding yeast) (Kondo et al., 2001; Melo et al., 2001; Rouse and Jackson, 2002b; Zou and Elledge, 2003). The presence of both intact complexes is required for efficient checkpoint signalling, possibly via an amplification loop. A candidate structure for the recruitment of these checkpoint protein complexes (and thus checkpoint activation) is extended regions of single-stranded DNA (ssDNA) (Lydall and Weinert, 1995; Zou and Elledge, 2003). The function of adaptor proteins such as Rad9 appears to be the physical linking of the activity of the checkpoint protein complexes at sites of DNA lesions (Mec1) to the activation of downstream checkpoint kinases that transmit the signal throughout the
nucleus (Rad53 and Chk1). This is consistent with the finding that Mec1 binds to DNA lesions, Rad9 is weakly recruited and Rad53 is not recruited (Kondo et al., 2001; Melo et al., 2001; Rouse and Jackson, 2002b; Zou and Elledge, 2003).

The adaptor role that Rad9 plays in signal transduction from Mec1 to Rad53 following DNA damage is well understood. Rad9 is hyper-phosphorylated in a MEC1-dependent manner after DNA damage (Emili, 1998; Sun et al., 1998; Vialard et al., 1998), and the phosphorylation of S/T[Q] consensus sites in Rad9 facilitates the recruitment and binding of Rad53 through its FHA (forkhead-associated) domains (Schwartz et al., 2002; Sun et al., 1998). Large phosphorylated Rad9 complexes are believed to act as ‘solid surface catalysts’ that facilitate a trans-autophosphorylation activity of Rad53 molecules (Gilbert et al., 2001). Phosphorylated (and activated) Rad53 molecules are then released by Rad9 to diffuse away and transmit the checkpoint signal throughout the nucleus. In budding yeast, some of the Rad53 downstream targets include the Dun1 protein kinase (de la Torre Ruiz and Lowndes, 2000; Gardner et al., 1999), the Cdc5 polo-like kinase (Sanchez et al., 1999) and Asf1, which is involved in chromatin remodelling (Emili et al., 2001; Hu et al., 2001).

The in vivo activation of Chk1 is also dependent on MEC1 and RAD9 (Sanchez et al., 1999). The role that Rad9 plays in the signal transduction from Mec1 to Chk1 is not well understood. Two reports have described a Rad9-Chk1 interaction by two-hybrid analysis in undamaged cells (Sanchez et al., 1999; Uetz et al., 2000) but other biochemical evidence for a physical interaction between Rad9 and Chk1 is lacking. Chk1 does not contain FHA domains and disruption of Mec1 phosphorylation sites in Rad9 does not abrogate the activation of Chk1 (Schwartz et al., 2002).

In fission yeast and mammalian cells, the mechanism by which Chk1 activity enforces a G2/M arrest are better characterized. Chk1 regulates Cdk (Cdc2) activity directly by inhibitory phosphorylation and also by targeting Cdc25, a negative regulator of Cdc2 (Rhind and Russell, 2000). In budding yeast, activation of the DNA damage checkpoint arrests cells at the metaphase-to-anaphase transition with high Cdk activity. The only downstream component of the Chk1 signalling pathway identified in *S. cerevisiae* to date is Pds1 (Securin) (Gardner et al., 1999; Sanchez et al., 1999; Wang et al., 2001).

Our aim was to understand more about how Rad9 functions in response to DNA damage. In this paper we describe a genetic screen to identify separation of function alleles in *rad9*. We identified a series of alleles defective in checkpoint signalling but proficient at maintaining the viability of *cdc13-1* mutants. By further characterizing these alleles we show that an N-terminal domain of Rad9 is required specifically for activation of the Chk1 kinase but not the Rad53 (Cds1/Chk2) kinase following DNA damage.

**Materials and Methods**

**Plasmid constructions**

A library of mutant *RAD9* alleles was generated using the GPS-LS Linker Scanning Kit (New England Biolabs). The procedure involved integrating a 1706 bp transposon into *RAD9* centromeric plasmids, and then removal of the transposon by digestion with the *Pme*I restriction enzyme to leave behind a 15 bp insertion. In the case of the *rad9*-1 allele, the *Pme*I digestion was unsuccessful and the transposon remained in the plasmid. A library of >24,300 linker insertions into a pRS416 *RAD9* plasmid was constructed (pDL767). The N-terminal truncation and internal deletion alleles were constructed by PCR cloning by introducing *NdeI* sites to generate alternative start sites. The *rad9A1-231* allele was cloned into pRS406 as a *NdeI*-BanHI fragment (pDL848).

**Strains**

All strains are in the W303 genetic background and are *RAD5*+. The *rad9A1-231* allele was subcloned from pDL848 into pRS406 as a *NotI*-XhoI fragment to create pDL901, and was integrated at the *URA3* locus of a rad9::LEU2 cdc13-1 cdc15-2 strain to generate DLY1908. The *rad9A1-231* allele was then crossed to other *rad9A* mutant strains.

**Drop testing**

Yeast strains were grown up to mid-log phase before a 1:5 dilution series was performed in either YEPD or SC-uracil media. Small drops of each series were spotted onto solid media plates using a 48-prong replica plating device (Sigma). Plates were incubated at appropriate temperatures for 4-5 days. In the rapid death assay, plates were subjected to a protocol of 36°C for 4 hours followed by 23°C for 4 hours, and this was repeated a further two times. After the completion of three cycles, the plates were incubated at 23°C.

**cdc13-1 arrest assay**

All strains carry *cdc13-1, cdc15-2* and *bar1*Δ mutations. Strains were grown to early log phase at 23°C before the addition of α-factor for 2.5 hours. After this period, the G1 arrest was observed by the presence of ‘schmoos’ by microscopy. Cultures were spun down and the cell pellets washed twice in YEPD at room temperature. Cells were then resuspended in 50 ml YEPD pre-warmed to 36°C and cultured at 36°C. Samples were removed at intervals of 20 minutes, fixed in ethanol, stained with DAPI (4¢,6-diamidino-2-phenylindole) and at least 100 cells scored by fluorescence microscopy (Lydall and Weinert, 1997). Cells with abnormal morphology were not scored.

**Immunoblotting**

Protein extracts were performed by glass bead breakage into TCA as previously described (Foiani et al., 1999) with minor adjustments. A sheep anti-Rad9 polyclonal antibody was generated using a His-tagged (pET22b) N-terminal half of Rad9 (*NdeI*-SacI fragment) as an antigen and the sera was affinity purified before use. The rabbit anti-Rad53 polyclonal antibody was a kind gift from Noel Lowndes. Mouse monoclonal 12CA5 (anti-HA) and 9E10 (anti-Myc) antibodies were used to detect epitope-tagged Chk1.

**Methylmethanesulphate (MMS) insult**

Cells harbouring the *cdc15-2* allele were grown to mid-log phase at 23°C before being shifted to 37°C for three hours. After three hours, MMS was added to a final concentration of 0.1% (v/v) and cells were cultured for a further 1 hour at 37°C. MMS was not added to mock-treated cells but they were otherwise cultured identically. Strains are DLY2191 (*RAD9 cdc15-2*), DLY2192 (*rad9::LEU2 cdc15-2*) and DLY2193 (*ura3::rad9A1-231:URA3 rad9::LEU2 cdc15-2*).

**Results**

A *RAD9* separation of function screen

*RAD9* has at least two functions in response to *cdc13-1*-induced telomeric DNA damage. First, *RAD9* is required to signal a cell-cycle arrest of *cdc13-1* cells at non-permissive temperatures (Weinert and Hartwell, 1993). Second, *RAD9* is
required to maintain the viability of cdc13-1 mutants at 36°C (Lydall and Weinert, 1995). To understand better the role of Rad9 in the DNA damage response, we sought to identify alleles of RAD9 that were defective in one or other of these functions but not defective in both.

CDC13 encodes an essential telomere-binding protein; in cdc13-1 mutants at non-permissive temperatures, the end-capping of telomeres appears to be compromised (Nugent et al., 1996). As a result, cdc13-1 cells accumulate ssDNA at their telomeres (Booth et al., 2001; Garvik et al., 1995; Lydall and Weinert, 1995). RAD9 is required for cell-cycle arrest of cdc13-1 mutants presumably because it mediates interactions between upstream and downstream checkpoint kinases. RAD9 also helps maintain the viability of cdc13-1 cells at 36°C by inhibiting nuclelease activity at telomeres, whereas other checkpoint genes, such as RAD24, promote nuclelease activity at telomeres (Booth et al., 2001; Lydall and Weinert, 1995).

Fig. 1A outlines the logic underlying two simple growth assays that measure different activities of RAD9. The ‘checkpoint arrest’ assay measures the ability of RAD9 alleles to inhibit growth of cdc13-1 mutants at the semi-permissive temperature of 27.3°C. cdc13-1 RAD9+ cells enter a checkpoint arrest and cannot form colonies at 27.3°C. By contrast, cdc13-1 rad9Δ cells are defective in checkpoint signalling and can form colonies at 27.3°C when low, presumably nonlethal, levels of DNA damage are present (Fig. 1B, rows 3,4). The 27.3°C temperature was determined empirically to maximize the difference in growth between cdc13-1 RAD9 and cdc13-1 rad9Δ cells (the temperature was set at 27.3°C but fluctuated between approximately 26.5 and 28°C). Therefore, by measuring growth of cdc13-1 mutants containing a library of RAD9 mutations at 27.3°C, we measure the ability of each RAD9 allele to activate a checkpoint-dependent cell-cycle arrest.

The ‘rapid death’ assay measures the ability of RAD9 alleles to maintain viability of cdc13-1 mutants incubated at 36°C for 4 hours. cdc13-1 RAD9 cells maintain good viability when incubated at 36°C for 4 hours (Fig. 1B, rows 1,2). By contrast, cdc13-1 rad9Δ mutants rapidly lose viability at 36°C, presumably as a result of the high levels of ssDNA induced under these conditions (Fig. 1B, rows 3,4). Therefore, by measuring viability of cdc13-1 mutants containing a library of RAD9 alleles after short incubations at 36°C, we presumably measure the ability of each RAD9 allele to inhibit nucleases at telomeres. Rapid loss of viability is not a result of failing to arrest because cdc13-1 rad24Δ cells are checkpoint defective but maintain viability as well as cdc13-1 RAD+ mutants (Lydall and Weinert, 1995).

Identification of potential separation of function alleles

To identify mutations in RAD9 that were specifically defective in checkpoint signalling or nuclelease inhibition, we generated a library of >23,000 rad9Δ alleles by linker scanning mutagenesis. This library was transformed into a cdc13-1 rad9Δ strain and ~3,600 transformants were individually tested in the

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Fig. 1. Genetic separation of function screen. (A) The ‘checkpoint arrest’ assay measures colony growth at the semi-permissive temperature of 27.3°C. In this assay, RAD9 cdc13-1 cells do not form colonies because they enter a G2/M arrest. By contrast, rad9Δ cdc13-1 cells cannot arrest and do form colonies. The rapid death assay measures colony growth after three periods of growth at the restrictive temperature of 36°C for 4 hours and finally a return to growth at 23°C. This assay RAD9 cdc13-1 cells retain viability and form colonies, whereas rad9Δ cdc13-1 cells lose viability in this assay. In practice, to distinguish better between the growth of RAD9+ and rad9Δ cells, plates were subjected to three 4-hour periods at the restrictive temperature of 36°C, separated by 4-hour periods of recovery at the permissive temperature of 23°C. Colonies were then allowed to form at 23°C. (B) Novel alleles rad9i-1 and rad9s-2 have opposite phenotypes in the two assays. pRS416 plasmids containing the RAD9 (lanes 1,2), rad9i-1 (lanes 5,6) and rad9s-2 (lanes 7,8) alleles were transformed into a rad9Δ cdc13-1 strain. An empty vector was transformed into lanes 3 and 4. A 1:5 dilution series was prepared and spotted onto SC-uracil (left two) or YEPD (right) plates, which were incubated as shown. (C) Wild-type RAD9 is 1309 amino acids, contains an [ST/IQ] cluster domain (SCD) and two BRCT domains. (D) The rad9i-1 allele contains an in-frame 5 amino acid insertion of GMFKH after C853. (E) The rad9s-2 allele contains a transposon insertion into the 5’ half of the RAD9 gene. The first 711 bp of the RAD9 coding sequence have been removed and replaced by 335 bp of transposon sequence (shown in black). The longest open reading frame remaining encodes an N-terminal truncation of Rad9 using the ATG at methionine M243 as a start codon.
checkpoint arrest and rapid death assays (Fig. 1A,B). Two classes of allele with phenotypes different to that of RAD9 and rad9Δ alleles were chosen for further analysis (Fig. 1B). One class permitted no colony growth in either the checkpoint arrest or rapid death assays (Fig. 1B, lanes 5,6). These alleles might encode Rad9 proteins that are proficient in checkpoint signalling but defective in nuclease inhibition and therefore we termed them rad9i (inhibition defective). We identified three weak and one strong rad9i alleles that we named rad9i-1 (Fig. 1B, lanes 5,6). A second class of allele permitted colony growth in both the checkpoint arrest and rapid death assays (Fig. 1B, lanes 7,8). These alleles might encode Rad9 proteins that are defective in checkpoint signalling but proficient in nuclease inhibition, and therefore we termed them rad9s (signalling defective). Approximately 10 rad9s alleles with differing strength phenotypes were identified.

Sequence analysis showed that the rad9i-1 allele encodes a full-length Rad9 with a 5 amino acid insertion after cysteine 853 (Fig. 1D). However, subsequent analysis of the rad9i-1 allele has determined that it encodes a temperature-sensitive allele of RAD9 that appears to be defective at both cell-cycle arrest and nuclease inhibition functions at 36°C (data not shown).

The rad9s-1 allele contained a 1.7 kb transposon insertion 711 bp into the RAD9 coding sequence (data not shown). The rad9s-2 allele shown is a derivative of rad9s-1 with the first 711 bp of RAD9 coding sequence and the majority of the transposon sequence removed (Fig. 1E). Sequence analysis demonstrated that no fusion proteins could be generated between the transposon and RAD9 sequences (data not shown). This suggested that the transposon sequences were activating expression of an N-terminal truncation of RAD9. The longest open reading frame (ORF) that remains in rad9s-2 uses the ATG encoding M243 as an alternative start codon.

**N-terminal truncations of Rad9 have a checkpoint signalling defect**

The phenotypes of the rad9s-1 and rad9s-2 alleles suggested that N-terminal truncations of Rad9 resulted in a loss of checkpoint signalling activity but retention of nuclease inhibition activity. To confirm this, we constructed a series of de novo N-terminal truncations of Rad9 on single copy plasmids. Three constructs that used the ATG codons encoding methionines M232, M243 and M358 of wild-type Rad9 as alternative start codons were generated. All three truncated Rad9 proteins were the predicted sizes by western blotting and had a clear rad9s phenotype (data not shown). This shows that the first 358 amino acids of Rad9 are not required for preventing rapid death of cdc13-1 mutants but contribute to signalling cell-cycle arrest in these mutants.

**Deletion analysis of RAD9**

To determine further which N-terminal amino acids of Rad9 are important for signalling cell-cycle arrest, we performed a deletion mapping analysis to identify the smallest deletion of RAD9 that retained the checkpoint-defective rad9s phenotype (Fig. 2A). Deletion of amino acids 1-39, or 201-231, did not affect Rad9 function in either the checkpoint arrest or the rapid death assays (Fig. 2A). However, deletion of amino acids in the 40-200 region resulted in weak rad9s phenotypes similar to those shown in rows 4-6 and 8-10. (B) The rad9D1-231 allele has the strongest rad9s phenotype when integrated; de novo deletion alleles of RAD9 were integrated at the URA3 locus of a rad9::LEU2 cdc13-1 cdc5-2 strain and tested in the two growth assays.
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was relatively poor, the alignment did reveal a 140LEDTPL 145

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Fig. 3. N-terminal domains of Rad9 are comparatively well conserved in other yeast species. Rad9 orthologues in two divergent budding yeasts (Saccharomyces castelli and Saccharomyces klayveri) were obtained from the Saccharomyces Genome Database. Amino acids 40-200 of the three Rad9 orthologues were aligned with amino acids 40-200 from S. pombe Crb2 using the ClustalW algorithm. Perfectly conserved residues are highlighted in black, structurally similar residues are highlighted in grey.

(Fig. 2A). Other small deletions within the 40-200 region also

resulted in weak rad9s phenotypes, leading us to conclude that

a comparatively large region of the first 200 amino acids of Rad9

was important for signalling cell-cycle arrest. We did not

measure expression levels of the various deletions so cannot

exclude the possibility that some of the rad9s phenotypes are a

result of altered protein abundance.

We had previously noted that our rad9s alleles had stronger

phenotypes when integrated compared with when expressed

from plasmids. We therefore integrated several novel rad9

alleles at the URA3 locus of a cdc13-1 strain. The rad9α-231

allele had a consistently stronger rad9s (signalling-defective) phenotype than deletions of 80 amino acids or a small F120HM

substitution (Fig. 2B), so the integrated rad9α-231 allele was used for further experiments.

To help determine which amino acids within the N-terminus of Rad9 are important for signalling cell-cycle arrest, we first

examined an alignment between S. cerevisiae Rad9 and its

putative orthologues in two divergent budding yeasts, S. castelli and S. klayveri [sequences from the Saccharomyces Genome Database (SGD) website (Cliffen et al., 2003)]. The primary amino acid sequence was not highly conserved

between these three species but some patches of sequence

showed high identity in the three proteins (Fig. 3, domains A

and B). We then took amino acids 40-200 from the three

Saccharomyces proteins and aligned them with Crb2, the S. pombe homologue. Although the overall level of homology was relatively poor, the alignment did reveal a 140LEDTP145 motif in S. cerevisiae Rad9 that is very well conserved in the two other budding yeasts and in S. pombe Crb2 (Fig. 3, domain B). However, when we repeated this alignment process with other ‘adaptor’ proteins, we did not find any significant alignment to Mrc1 (S. cerevisiae), xClaspin (Xenopus),

BRCA1 (human) or p53BP1 (human) (data not shown).

mutants have a chk1Δ-like phenotype

The checkpoint signalling function of Rad9 in response to

cdc13-1-induced damage is mediated through the downstream

kinases Rad53 and Chk1, acting in parallel pathways (Gardner

et al., 1999; Sanchez et al., 1999). We hypothesized that

rad9Δ-231 mutants might be defective in one or both of these

parallel signalling branches. To test this, we examined

phenotypes that distinguish between chk1 and rad53 mutants.

To test whether rad9Δ-231 mutants were defective in RAD53- and/or CHK1-dependent signalling pathways, we first measured viability after ultraviolet (UV) irradiation. In budding yeast, chk1Δ mutants are not UV sensitive but rad53 mutants are UV sensitive (Allen et al., 1994; Liu et al., 2000; Sanchez et al., 1996).

Fig. 4A shows that rad9Δ-231 cells are as UV resistant as wild-type and chk1Δ cells. By contrast, rad53Δ cells are UV

sensitive, but not as sensitive as rad9Δ cells. In our strain

background, we consistently did not observe any enhanced UV

sensitivity of rad53Δ chk1Δ double mutants compared with

rad53Δ single mutants (Fig. 4A).

The lack of UV sensitivity of rad9Δ-231 mutants suggested

that the RAD53 pathway was intact in these cells; however, this

experiment did not provide any information on whether the

CHK1 pathway was also intact. To address this issue we

combined the rad9Δ-231 allele with a yku70Δ mutation.

Ku is a conserved hetero-dimeric protein involved in non-

homologous end-joining repair and also binds to telomeres in

yeast and mammalian cells (Baumann and Cech, 2000; Haber,

1999; Polonianska et al., 1998; Porter et al., 1996). Deletion of

the yku70 mutant cells makes telomeres less sensitive. At 37°C,

yku70Δ cells accumulate ssDNA at their telomeres and enter a

G2/M arrest dependent on RAD9 and CHK1 but independent of

RAD53 and DUN1 (Maringele and Lydall, 2002) (Fig. 4B).

If rad9Δ-231 mutants were unable to activate the CHK1-dependent pathway to arrest cell division in yku70Δ mutants, they should, like chk1Δ mutants, permit growth of yku70Δ mutants at 37°C. Fig. 4B shows that yku70 rad9Δ-231 double mutants grew as well at 37°C as yku70Δ rad53Δ mutants, and grew similarly to yku70Δ chk1Δ mutants. By contrast, yku70Δ rad53Δ and yku70Δ dun1Δ strains grew poorly at 37°C. These data strongly suggested that the CHK1 signalling pathway was abrogated in rad9Δ-231 mutants.

To gain further evidence that the CHK1 signalling pathway was specifically defective in rad9Δ-231 mutants, we measured the cell-cycle arrest kinetics of cdc13-1 mutants. At 36°C, rad9Δ cdc13-1 cells have a complete checkpoint defect, whereas dun1Δ cdc13-1, rad53Δ cdc13-1 or chk1Δ cdc13-1 are only partially checkpoint defective (Gardner et al., 1999;
Sanchez et al., 1999; Schwartz et al., 2002). To measure the checkpoint defect of rad9Δ1-231 strains, bar1 and cdc15-2 mutations were used to quantify the fraction of cdc13-1 mutant cells that had failed to arrest during a single cell cycle (Lydall and Weinert, 1997).

When released from α-factor at 36°C, rad9Δ cdc13-1 cdc15-2 cells begin to accumulate at telophase (the checkpoint-independent cdc15-2 arrest point) within 80-100 minutes (Fig. 4C). By contrast, chk1Δ cdc13-1 cdc15-2 cells reach telophase later, at 120-140 minutes, and dun1Δ cdc13-1 cdc15-2 later still at 200-240 minutes (Fig. 4C). rad9Δ1-231 cdc13-1 cdc15-2 strains are not as checkpoint defective as rad9Δ strains, but they reach late nuclear division with faster kinetics than chk1Δ mutants. Thus, the phenotype of rad9Δ1-231 mutation is most similar to chk1Δ rather than dun1Δ mutations in causing partial checkpoint arrest in response to cdc13-1-induced damage.

In summary, analysis of the UV sensitivity, and cell-cycle arrest after yku70Δ- and cdc13-1-induced damage strongly suggests that rad9Δ1-231 cells are specifically defective in the CHK1-PDS1 branch of checkpoint control rather than the RAD53-DUN1 pathway.

MMS-induced Chk1 phosphorylation is defective in rad9Δ1-231 cells

To gain biochemical support for the idea that rad9Δ1-231 cells are specifically defective in activating the Chk1-dependent checkpoint signalling pathway, we examined phosphorylation of Rad9, Chk1 and Rad53 following genotoxic insult with the alkylating agent methylmethanesulphate (MMS). To avoid the complication that RAD9-independent phosphorylation of Rad53 is observed in asynchronous cultures (Pellicioli et al., 1999), we arrested the cultures in telophase using the cdc15-2 conditional allele prior to MMS treatment. At the cdc15-2 arrest point, MMS-induced phosphorylation of Rad53 and Chk1 is totally dependent on RAD9 (Schwartz et al., 2002).

Following treatment with the DNA-alkylating agent MMS, the truncated Rad9Δ1-231 protein was phosphorylated to apparently the same extent as the wild-type Rad9 protein (Fig. 5). MMS-induced phosphorylation of Rad53 occurred in both RAD9 and rad9Δ1-231 cells but not in rad9Δ mutants (Fig. 5). We noted that the extent of the Rad53 phosphorylation is slightly greater in RAD9 cells compared with rad9Δ1-231 cells. By contrast, epitope-tagged Chk1 was clearly phosphorylated in RAD9 cells but this phosphorylation is defective in rad9Δ1-231 cells and rad9Δ cells (Fig. 5). Therefore, rad9Δ1-231 cells are specifically defective at phosphorylating Chk1 but not Rad53 in response to MMS-induced DNA damage.

Discussion

DNA damage responsive checkpoint pathways are exquisitely sensitive signal transduction cascades that can inhibit cell division in response to single double-strand breaks. As in many other signal transduction cascades, kinases lie at the heart of checkpoint pathways. Adaptor/mediator proteins, which facilitate interactions between upstream and downstream checkpoint kinases, are also essential for checkpoint signal transduction. In this paper, we have sought to understand the role of the budding yeast Rad9 adaptor protein in facilitating interactions between upstream and downstream checkpoint kinases.

Rad9 mediates interactions between the upstream kinase Mec1 and two parallel downstream kinases Rad53 and Chk1. The mechanism by which Rad9 facilitates interactions between

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**Fig. 4.** rad9Δ1-231 mutants have similar phenotypes to chk1Δ mutants. Strains of the indicated genotypes were grown in liquid culture before a fivefold dilution series was prepared and spotted to YEPD plates. Strain numbers are in parenthesis. (A) rad9Δ1-231 mutants are not UV sensitive. Plates were untreated or exposed to 40 J/m² UV before incubation for three days at 30°C. (B) yku70Δ rad9Δ1-231 cells are checkpoint arrest defective at restrictive temperatures. Growth was measured at the permissive temperature of 30°C and the restrictive temperature of 37°C. (C) Arrest kinetics of cdc13-1 mutants in synchronous cultures. Checkpoint mutations were combined with the cdc13-1 and cdc15-2 mutations. Cultures were arrested with α-factor at 23°C then released into fresh media at 36°C. Accumulation of cells at mid-nuclear division (cdc13-1 arrest point) and late nuclear division (cdc15-2 arrest point) was measured by fluorescence microscopy at intervals of 20 minutes.
Mutants have a partial checkpoint defect in response to DNA damage that is most like a CDC15-2-induced damage, but the magnitude of the signalling response is stronger than that seen with DNA damage. The strongest mutant phenotype was observed with two separate extracts and Chk1 phosphorylation was detected with both HA- and Myc-tagged proteins.

We mapped the Chk1 activation domain by deletion analysis. The strongest mutant phenotype was observed with the rad9Δ231 truncation allele, and the implication is that more than one residue or motif within the 40-200 amino acid region are required for Chk1 activation. This is supported by the fact that several motifs seem to be well conserved across three Saccharomyces species against a background of reasonably weak sequence identity (Fig. 3). The 40-200 amino acid region does not contain any Mec1 phosphorylation target sites but interestingly does contain three potential Cdk (cyclin-dependent kinase) phosphorylation sites (Toh and Lowndes, 2003). Rad9 phosphorylation in undamaged cells is cell-cycle regulated (Vialard et al., 1998); in addition, the activity of the fission yeast Crb2 protein is also regulated by Cdk phosphorylation (Caspari et al., 2002; Esashi and Yanagida, 1999). The question of whether Cdk (Cdc28)-dependent phosphorylation somehow regulates the Rad9-Chk1 checkpoint signalling pathway is therefore an interesting line of further enquiry. We note that there is a conserved potential phosphorylation site (DTP) in motif B of Rad9 and its orthologues (Fig. 3).

In summary, the data presented here supports the view that Rad9 activates Rad53 and Chk1 by distinct and independent mechanisms (Schwartz et al., 2002). This is consistent with the fact that phosphopeptide-binding FHA domains in Rad53 mediate its interaction with Rad9 and that these domains are absent in Chk1.

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