The novel DNA damage checkpoint protein Ddc1p is phosphorylated periodically during the cell cycle and in response to DNA damage in budding yeast

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The DDC1 gene was identified, together with MEC3 and other checkpoint genes, during a screening for mutations causing synthetic lethality when combined with a conditional allele altering DNA primase. Deletion of DDC1 causes sensitivity to UV radiation, methyl methanesulfonate (MMS) and hydroxyurea (HU). ddc1Δ mutants are defective in delaying G1→S and G2→M transition and in slowing down the rate of DNA synthesis when DNA is damaged during G1, G2 or S phase, respectively. Therefore, DDC1 is involved in all the known DNA damage checkpoints. Conversely, Ddc1p is not required for delaying entry into mitosis when DNA synthesis is inhibited. ddc1Δ and mec3Δ mutants belong to the same epistasis group, and DDC1 overexpression can partially suppress MMS and HU sensitivity of mec3Δ strains, as well as their checkpoint defects. Moreover, Ddc1p is phosphorylated periodically during a normal cell cycle and becomes hyperphosphorylated in response to DNA damage. Both phosphorylation events are at least partially dependent on a functional MEC3 gene.

Keywords: budding yeast/checkpoints/DNA damage/phosphorylation

Introduction

Cell proliferation is dependent on the ordered completion of two key events during the mitotic cell cycle: genome replication during S phase and segregation of the duplicated genomes during mitosis. A complex network of surveillance mechanisms, called checkpoints, delays cell cycle progression when DNA is damaged or incompletely replicated, or when the mitotic spindle is not assembled properly, probably allowing time for DNA repair and replication before entry into mitosis and for the alignment of chromosomes on the spindle before initiation of anaphase (for reviews, see Hartwell and Weinert, 1989; Murray, 1994, 1995; Friedberg et al., 1995; Elledge, 1996; Paulovitch et al., 1997b). Defective checkpoint controls may play an important role in the genesis of cancer cells, allowing rapid accumulation of genetic changes (Hartwell and Kastan, 1994). For example, mutations in the p53 tumor suppressor gene or in the ataxia telangiectasia ATM gene, both involved in the response to DNA damage in human cells, are often associated with cancer, probably due to increased genomic instability and mutagenesis (reviewed in Enoch and Norbury, 1995). Several data indicate that the basic mechanisms controlling the response to DNA damage are conserved in all eukaryotic cell types, even if different organisms seem to have adapted them in different ways (reviewed in Carr and Hoekstra, 1995; Elledge, 1996; Lydall and Weinert, 1996; Carr, 1997). Studies in simple model systems, such as the evolutionarily distant yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe, have allowed the identification of many checkpoint proteins, several of which have structural and functional equivalents in man, and provide an important contribution to the understanding of the biochemical basis of checkpoint controls in all eukaryotes.

In the budding yeast S.cerevisiae, at least three DNA damage checkpoints have been identified, which inhibit the G1→S transition (G1/S checkpoint) (Siede et al., 1993, 1994), slow down progression through S phase (intra-S checkpoint) (Paulovitch and Hartwell, 1995) and delay the G2→M transition (G2/M checkpoint) (Weinert and Hartwell, 1988), when DNA is damaged during G1, S or G2 phase, respectively. Several genes are known to be involved in these control mechanisms. The MEC1 and RAD53 essential gene products must play pivotal roles in different signal transduction pathways, since they are required not only for proper response to DNA damage, but also for the S/M checkpoint preventing entry into mitosis when S phase is inhibited by the ribonucleotide reductase inhibitor hydroxyurea (HU) (Zheng et al., 1993; Allen et al., 1994; Weinert et al., 1994; Sanchez et al., 1996; Siede et al., 1996; Sun et al., 1996). The non-essential genes RAD9, RAD17, RAD24 and MEC3 are required for all the known DNA damage checkpoints, but not for delaying entry into mitosis when S phase is inhibited (Siede et al., 1993, 1994; Weinert and Hartwell, 1993; Weinert et al., 1994; Longhese et al., 1996a; Paulovitch et al., 1997a). Moreover, both the large subunit of replication protein A (RPA) and the catalytic subunit of DNA primase are involved in a subset of DNA damage checkpoints, i.e. the G1/S and intra-S checkpoints (Longhese et al., 1996b; Marini et al., 1997), while the S/M checkpoint requires DNA polymerase ε (pol ε), the large subunit of replication factor C (RF-C) and the DPB11 gene product (a protein interacting with pol ε) (Araki et al., 1995; Navas et al., 1995; Sugimoto et al., 1996). Finally, several checkpoint genes have different roles in transcriptional induction following DNA damage (Aboussekra et al., 1996; Kiser and Weinert, 1996; Navas et al., 1996).

Recent data indicate that the RAD9 and RAD24 gene products are both required for processing single-stranded subtelomeric DNA regions, which accumulate in cdc13 temperature-sensitive mutant cells at non-permissive temperature (Garvik et al., 1995; Lydall and Weinert, 1995,
MEC3, RAD17 and RAD24 belong to the same epistasis group, while RAD9 is in a class on its own and acts in opposition to RAD24 after cdc13-induced damage (Lydall and Weinert, 1995). Since Rad17p shows similarity to a 3’–5’ DNA exonuclease, it has been proposed that Rad17p, Rad24p and Mec3p control degradation of DNA after Cdc13p inactivation, and that the role of Rad9p is to inhibit this degradation (Lydall and Weinert, 1995, 1996). The involvement of these four checkpoint proteins in DNA metabolism suggests that they might act close to the primary DNA damage event, but the molecular mechanisms linking DNA damage recognition, processing and repair to cell cycle arrest are still obscure, and other factors and interactions are likely to be involved.

Here we describe a new gene, DDC1, whose deletion causes sensitivity to UV radiation, methyl methanesulfonate (MMS) and HU comparable with that observed in mec3Δ strains. We show that DDC1 and MEC3 genes belong to the same epistasis group and DDC1 function is required to delay cell cycle progression when DNA is damaged during G1, S or G2 phase, but not to block S–M transition when S phase is inhibited by HU. Furthermore, Ddc1p is phosphorylated periodically during a normal cell cycle and hyperphosphorylated in response to DNA damage. MEC3 is required for proper phosphorylation of Ddc1p, and DDC1 overexpression partially compensates the checkpoint defects of mec3Δ strains.

Results

Cloning and disruption of the DDC1 gene

A genetic screening for mutations causing synthetic lethality when combined with the pri1-2 cold-sensitive allele, altering the catalytic subunit of DNA primase, allowed the identification of a number of independent mutations belonging to seven complementation groups, possibly corresponding to seven different genes, that we named PIP1–7 (Longhese et al., 1996a). Some of these mutations caused additional phenotypes, like hypersensitivity to UV radiation, MMS and HU, suggesting some function of the corresponding gene products in DNA repair and/or checkpoint mechanisms. Cloning of the PIP3 gene allowed the establishment that it is in fact the MEC3 DNA damage checkpoint gene (Longhese et al., 1996a). Transformation of the remaining pri1-2 pip double mutants with centromeric plasmids carrying the MEC1, RAD53, RAD17 and RAD24 genes showed that synthetic lethality due to pip1 pri1-2 and pip7 pri1-2 combinations was fully compensated by MEC1 and RAD24, respectively. The identity of PIP1 with MEC1 was confirmed by an allelism test (data not shown). The synthetic lethal effect due to combination of the pri1-2 allele with the pip2, pip4, pip5 and pip6 mutations could not be complemented by any of the checkpoint genes analyzed.

Cloning of the PIP5 gene was achieved by screening a yeast genomic DNA library constructed in a LEU2 centromeric plasmid (Jansen et al., 1993) for complementation of the pri1-2 pip5-1 synthetic lethal phenotype (see Materials and methods). Sequencing of ~300 nucleotides from both ends of the smallest yeast DNA insert identified by this screening and a search of the yeast genome database revealed that the cloned fragment was located on S.cerevisiae chromosome XVI, between positions 175 452 and 186 891. Further analysis allowed us to establish that a 2772 bp NsiI–BsrI DNA fragment (Figure 1A) was sufficient to complement the pri1-2 pip5-1 synthetic lethal phenotype. This fragment contained only one complete open reading frame (ORF), YPL194w (nucleotides 179 276–181 111; accession No. U212C1), whose deletion –5217 from the translation initiation codon, giving rise to the ddc1Δ allele. (B) Serial dilutions of YPD-saturated cell cultures of strains YLL231 (1) and YLL245 (2) were spotted on SC plates without (+) or with (−) 5-FOA, to assay the ability of the two strains to lose the URA3 centromeric plasmid carrying the PRI1 allele [URA3 PRI1]. (C) Serial dilutions of YPD-saturated cell cultures of strains K699 (wt) and YLL244 (Δ) were spotted on YPD plates without (YPD) or with MMS (0.01%) or HU (150 mM). YPD plates were made in duplicate and one of them was UV irradiated (30 J/m²) (UV).

Substitution of most of the DDC1 chromosomal ORF with the heterologous KanMX4 cassette gave rise to the ddc1Δ allele (see Figure 1A and Materials and methods) that was not lethal in PRI1 cells, while pri1-2 ddc1Δ strains were inviable (Figure 1B). The cell viability of the
pol1-l and rfa1-M2 DNA replication mutants (Longhese et al., 1996a) was also severely affected by the ddc1Δ mutation (data not shown). Finally, like the original pip5-l mutant (Longhese et al., 1996a), ddc1Δ strains were more sensitive than wild-type to UV, MMS and HU (Figure 1C).

**The DDC1 gene belongs to the MEC3 epistasis group and is involved in all the known DNA damage checkpoints**

As shown in Figure 2, strains carrying the single ddc1Δ or mec3Δ alleles showed very similar sensitivity to UV radiation, which was indistinguishable from that of a ddc1Δ mec3Δ double mutant, indicating that the DDC1 and MEC3 genes belong to the same epistasis group. The ddc1Δ rad9Δ strain was instead more sensitive to UV than was the single mutant (Figure 2), similarly to what was observed previously for mec3Δ rad9Δ double mutants (Lydall and Weinert, 1995; Longhese et al., 1996a). Therefore, DDC1 belongs to the RAD24 epistasis group, that also includes the MEC3 and RAD17 genes (Lydall and Weinert, 1995), while RAD9 represents a different group.

As shown in Figure 3A, ddc1Δ cells are defective in delaying G1–S transition after UV irradiation in G1. In fact, when ddc1Δ α-factor-arrested cell cultures were UV irradiated and then released from G1 block, both progression through S phase (Figure 3A, top) and budding kinetics (Figure 3A, bottom) were much faster than in wild-type cell cultures under the same conditions. Cell survival after UV treatment was lower in ddc1Δ cell cultures than in wild-type (12 and 58%, respectively). ddc1Δ cell viability did not increase when cell cycle progression was delayed by holding the UV-irradiated cultures in G1 by α-factor for 120 min (data not shown).

A similar behavior was also observed previously in strains carrying null alleles of RAD9 (Siede et al., 1993) and MEC3 (our unpublished observation) checkpoint genes, for which a direct involvement in DNA repair has been suggested (Lydall and Weinert, 1995).

Slowing down the rate of DNA synthesis when DNA is damaged during S phase is a genetically controlled process (Paulovich and Hartwell, 1995), and the data in Figure 3B show that Ddc1p is involved in this checkpoint mechanism. In fact, α-factor-synchronized ddc1Δ cells, when released from G1 arrest in the presence of MMS, mostly reached a 2C DNA content within 45 min (Figure 3B), while wild-type cell cultures under the same conditions progressed through S phase very slowly, reaching a 2C DNA content only after 180 min. MMS-treated ddc1Δ cells progressively lost viability during the experiment (25, 12 and 0.4% cell survival after 30, 60 and 180 min of MMS treatment, respectively), while the MMS concentration used did not substantially affect wild-type cell survival throughout the experiment. Therefore, Ddc1p is needed for the intra-S control mechanism that requires all the checkpoint proteins analyzed so far (Paulovich and Hartwell, 1995; Longhese et al., 1996a,b; Navas et al., 1996; Marini et al., 1997; Paulovich et al., 1997a).

A functional DDC1 gene product is also essential for properly delaying the G2/M transition when DNA is damaged in G2 (Figure 3C). In fact, when cell cultures were released from nocodazole arrest after UV irradiation, the appearance of binucleate cells in wild-type cultures was appreciably delayed compared with the unirradiated control, while ddc1Δ cells went through nuclear division much faster than wild-type. ddc1Δ cell survival after UV treatment was much lower than that of wild-type cells under the same conditions (15 and 82%, respectively). As already observed when cells were irradiated in G1, ddc1Δ cell viability was not increased by holding the cultures in nocodazole for 120 min after UV irradiation in G2 (data not shown), again suggesting a direct involvement of Ddc1p in DNA repair.

Based on the above results, the DDC1 gene product is required for all the known DNA damage checkpoints. Conversely, Ddc1p does not appear to be involved in the control mechanism coupling completion of S phase to entry into mitosis, since ddc1Δ cells properly arrest with a single nucleus and short spindles after S phase block by HU treatment (data not shown). The small, but significant, increase in HU sensitivity of ddc1Δ strains compared with wild-type (Figure 1C), which is similar to that observed for other DNA damage checkpoint mutants with a proficient S/M checkpoint (Weinert et al., 1994; Longhese et al., 1996a), must therefore be related to something other than defective cell cycle arrest in response to incomplete DNA replication.

As previously observed for other DNA damage checkpoint mutants (Longhese et al., 1996; Paulovich et al., 1997a), ddc1Δ cells still show some delay in cell cycle progression after DNA damage in G1, G2 or S phase compared with untreated cells, suggesting that an as yet unidentified DDC1-independent pathway(s) might contribute to these responses.

**DDC1 overexpression can partially suppress sensitivity to MMS and HU of mec3Δ mutants, as well as their intra-S checkpoint defect**

Since ddc1Δ and mec3Δ mutants belong to the same epistasis group and exhibit very similar phenotypes, we examined the effect of overexpressing DDC1 in a mec3Δ background. For this purpose, the DDC1 ORF was fused to the galactose-inducible GAL1 promoter and a single
Fig. 3. A functional DDC1 gene is required for all the known DNA damage checkpoints. Strains were K699 (wt) and YLL244 (ddc1Δ) and times are given in minutes. (A) α-Factor-synchronized cultures were UV irradiated (40 J/m²) and released from α-factor at time zero. FACS analysis of unirradiated (−) and irradiated (+) cultures at the indicated times after α-factor release (time zero) is shown in the top part of the panel, while the bottom part shows the percentage of budded cells in both unirradiated (open symbols) and irradiated (closed symbols) cultures. (B) α-Factor-synchronized cultures were released from α-factor at time zero, either in YPD or in YPD containing 0.02% MMS. Untreated (−) or MMS-treated (+) samples were taken at the indicated times after α-factor release (time zero) and analyzed by FACS (black histograms). Overlayed histograms represent the cell cycle distributions of the asynchronous cultures. (C) Cell cultures were arrested with nocodazole and were UV irradiated (50 J/m²). Cell cycle progression was monitored at the indicated times in unirradiated (open symbols) and UV-irradiated (closed symbols) cultures after release from nocodazole, by direct visualization of nuclear division using DAPI staining.
copy of the GAL1–DDC1 gene fusion was integrated at the LEU2 locus of otherwise isogenic wild-type and mec3Δ strains (see Materials and methods). DDC1 overexpression, which did not cause any detectable phenotype in the wild-type background, partially suppressed MMS sensitivity and, to a lower extent, HU sensitivity of the mec3Δ strain (Figure 4).

As shown in Figure 5A, mec3Δ GAL1-DDC1 cell cultures, synchronized with α-factor and then released from the G1 block in the presence of MMS under galactose-induced conditions, progressed through S phase more slowly than similarly treated mec3Δ cell cultures. Furthermore, cell survival following MMS treatment was higher in mec3Δ GAL1-DDC1 than in mec3Δ cell cultures (Figure 5B). Therefore, high levels of Ddc1p can partially suppress the intra-S checkpoint defect of the mec3Δ mutant. DDC1 overexpression in wild-type cells did not cause any significant effect on response to MMS treatment during S phase (Figure 5A and B). When a similar experiment was carried out by comparing wild-type, GAL1-MEC3, ddc1Δ and ddc1Δ GAL1-MEC3 cell cultures (see Table I and Materials and methods), MEC3 overexpression did not suppress the MMS sensitivity of the ddc1Δ strain and had no effect on the rate of DNA synthesis in any genetic background, neither in the absence nor in the presence of MMS (data not shown).

**Ddc1p is phosphorylated periodically during the cell cycle and in response to DNA damage**

In order to characterize the DDC1 gene product, we constructed a fully functional copy of the gene, expressing a 2HA-tagged Ddc1p, that was used to generate strain YLL334, carrying the 2HA-DDC1 allele at the DDC1 chromosomal locus (see Materials and methods). As shown in Figure 6B, when anti-HA antibodies were used on Western blots of crude extracts prepared from exponentially growing YLL334 cells, they specifically detected two major bands that did not appear in extracts prepared from the isogenic strain carrying the untagged DDC1 allele, therefore identifying Ddc1p. While the faster migrating band was present throughout the whole cell cycle, the slower migrating band was not present in α-factor-arrested wild-type cells and accumulated periodically during the cell cycle, increasing in level throughout S phase (Figure 6B and C) and decreasing concomitantly with the appearance of binucleate cells (Figure 6A). Therefore, Ddc1p is subject to cell cycle-dependent post-translational modification(s). When a similar experiment was performed in
Ddc1p and DNA damage checkpoints

Fig. 6. Ddc1p is modified periodically during the cell cycle in wild-type, but not in mec3Δ cells. Exponentially growing (exp) YLL334 (wt) and YLL335 (mec3Δ) cells, expressing 2HA-Ddc1p from the DDC1 promoter, were synchronized with α-factor and released at time zero. (A) The percentage of budded (open symbols) and binucleate cells (closed symbols) was monitored at the indicated times. (B) At the same times, protein extracts were prepared and analyzed by Western blot with 12CA5 antibody, together with K699 cell extract containing only untagged Ddc1p (-HA). Protein bands corresponding to Ddc1p are indicated by brackets. (C) FACS analysis at the indicated times after α-factor release.

a mec3Δ strain carrying the 2HA-DDC1 allele, only the faster migrating Ddc1p form could be detected throughout the whole cell cycle (Figure 6B), indicating that post-translational modification of Ddc1p depends on functional Mec3p.

As shown in Figure 7A, treatment of wild-type cells with UV and MMS caused accumulation of a modified form of Ddc1p, which migrated more slowly than the retarded protein species observed in untreated S phase cells. The observed Ddc1p modification was at least partially MEC3 dependent. In fact, Ddc1p was predominantly unmodified in UV- and MMS-treated mec3Δ cells, and the small amount of modified protein observed in mec3Δ protein extracts migrated faster than the form detected in extracts prepared from similarly treated wild-type cells (Figure 7A). The observed changes in Ddc1p electrophoretic mobility were shown to be due to phosphorylation events (Figure 7B). In fact, the slower migrating protein species in both MMS-treated and S phase cell extracts was converted to the fastest migrating form by treatment with bacteriophage λ phosphatase.

HU treatment of wild-type cells caused the accumulation of a Ddc1p form with electrophoretic mobility indistinguishable from that accumulated during normal S phase (Figure 7A). By considering that Ddc1p is not required for HU-induced cell division arrest, while it is required for DNA damage response, Ddc1p hyperphosphorylation appears to correlate with Ddc1p checkpoint function.

In order to better define the kinetics of Ddc1p phosphorylation in response to DNA damage and its dependence on MEC3, α-factor-arrested cells were UV irradiated and Ddc1p was analyzed by Western blot after release from α-factor block. As shown in Figure 7C, hyperphosphorylated Ddc1p in UV-treated wild-type cells appeared
immediately after release from α-factor, it became the most abundant form in ~45 min, when most cells were unbudded (data not shown) with a 1C DNA content (Figure 7C, bottom), and it was then maintained until the end of the experiment. Both the kinetics and extent of Ddc1p phosphorylation in response to DNA damage in G1 were at least partially dependent on MEC3. In fact, when mec3Δ cells were UV irradiated in G1, Ddc1p phosphorylation was delayed by 20–30 min compared with wild-type (Figure 7C), although mec3Δ cells progressed through the cell cycle after α-factor release much faster than did wild-type cells under the same conditions (Figure 7C, bottom). Moreover, both the total amount of modified protein and the extent of modification, as judged by the changes in electrophoretic mobility, were reduced in UV-irradiated mec3Δ cells compared with wild-type (Figure 7C). No Ddc1p phosphorylation was observed in extracts prepared from wild-type cells that were kept in the presence of α-factor for 2 h after UV treatment in G1 (data not shown).

Similarly to what was observed after UV irradiation in G1, Ddc1p was also hyperphosphorylated in response to DNA damage in G2 (Figure 8). In fact, while nocodazole-arrested unirradiated cells contained only unphosphorylated Ddc1p, the hyperphosphorylated form of Ddc1p was detectable immediately after release from nocodazole arrest of UV-treated wild-type cells (Figure 8C), when most cells still contained undivided nuclei (Figure 8B). This response to UV-induced damage does not require cell cycle progression, since an identical extent of Ddc1p phosphorylation was observed in wild-type cells either released from nocodazole or kept for 2 h in the presence of the drug after UV treatment in G2 (Figure 8D). As expected, Ddc1p was instead phosphorylated only during S phase when cells were released from nocodazole arrest in the absence of DNA damage, and again this modification was MEC3 dependent (Figure 8A and C).

Ddc1p phosphorylation in response to DNA damage in G2 is also at least partially dependent on MEC3. In fact, only a small amount of partially modified Ddc1p was detectable in mec3Δ cells either released from nocodazole or kept in the presence of the drug after UV irradiation in G2 (Figure 8C and D). Therefore, the difference in Ddc1p phosphorylation between UV-treated wild-type and mec3Δ cells was not due to different kinetics of cell cycle progression.

**Discussion**

Response to DNA damage in eukaryotic cells involves specific surveillance mechanisms, which are genetically controlled and are essential for accurate transmission of genetic information during cell proliferation. In *S. cerevisiae*, the RAD9, RAD17, RAD24 and MEC3 gene products are all required for these processes and are proposed to act in concert, although with different roles, in processing DNA lesions, thus generating signals that arrest or slow down cell cycle progression in the presence of DNA damage (reviewed in Lydall and Weinert, 1996).

**Role of DDC1 in checkpoint and DNA repair mechanisms**

The previously uncharacterized DDC1 gene product is involved in all the known surveillance mechanisms controlling cell response to DNA damage. In fact, null *ddc1Δ* mutants, besides being more sensitive than wild-type to UV, MMS and HU, are defective in delaying G1–S and G2–M transition and in slowing down the rate of DNA synthesis when DNA is damaged during G1, G2 or S phase, respectively. Conversely, Ddc1p is not required for delaying entry into mitosis when DNA synthesis is inhibited by HU. As previously suggested for other DNA damage checkpoint genes (Siede *et al.*, 1993; Lydall and Weinert, 1995), DDC1 function is likely to be required for DNA damage processing/repair events, since lethality...
of G₁ or G₂ UV-irradiated ddc1Δ cells is not rescued by artificially arresting the cell cycle with α-factor or nocodazole, respectively.

Why ddc1 mutants, as well as mec3, rad24 and rad17 mutants, are more sensitive to HU than wild-type, even though these factors are not involved directly in cell cycle arrest in response to HU treatment (Weinert and Hartwell, 1993; Weinert et al., 1994; Longhese et al., 1996a; this work), is still an open question. Since ddc1 and mec3 mutations are synthetic lethal with pri1 mutations and severely affect cell viability of other DNA replication mutants at the permissive temperature (Longhese et al., 1996a; this work), Ddc1p and Mec3p might be involved specifically in sensing/processing altered DNA molecules arising from defective DNA replication, and HU might cause similar effects by interfering with DNA synthesis.

The Ddc1p amino acid sequence shows some homology with the product of the S.pombe rad9 gene, which is also involved in DNA damage checkpoints (Al-Khodairy and Carr, 1992; Enoch et al., 1992). Since the two yeast proteins are structurally related and functionally share similarities, they might have diverged from the same protein. The recently identified human Rad9 protein (Lieberman et al., 1996) is also related, but seems to have more similarities to the S.pombe gene product. As there is no obvious enzymatic activity associated with these proteins, it is possible that this reflects more flexibility of structural divergence.

**Ddc1p is phosphorylated periodically during the cell cycle and is hyperphosphorylated in response to DNA damage**

A phosphorylated form of Ddc1p appears periodically during the cell cycle, reaching the maximum level when most cells are in S phase and decreasing concomitantly with nuclear division. The event(s) leading to dephosphorylation or degradation of the phosphorylated form do not require nuclear division, since the phosphorylated Ddc1p is not detectable in nocodazole-arrested cells. The correlation between Ddc1p phosphorylation and progression through S phase suggests that the signal leading to this modification might be intrinsic to the DNA replication process. Since DDC1 is likely to be involved, together with the other RAD24 group genes, in processing single-stranded DNA lesions, its S phase-dependent phosphorylation might result from sensing single-stranded replication intermediates and/or spontaneous errors arising during DNA replication. This, in turn, might result in potentially active Ddc1p, that would then be required if accumulation of DNA lesions rises above the physiological level. Thus, Ddc1p phosphorylation is not expected to take place in undamaged nocodazole-arrested cells since, once DNA replication has been completed properly, there should be no more signals leading to Ddc1p phosphorylation.

When wild-type cells are UV irradiated in either G₁ or G₂, Ddc1p is hyperphosphorylated. This modification takes place in G₂ cells even if they are held in nocodazole after irradiation, and in unbudded cells with 1C DNA content following DNA damage in G₁. Therefore, Ddc1p hyperphosphorylation correlates with DNA damage response and does not require ongoing DNA synthesis.

**Functional interactions between DDC1 and MEC3**

The RAD9, RAD17, RAD24 and MEC3 genes have all been implicated in processing cdc13-induced lesions near to the telomeres (Garvik et al., 1995; Lydall and Weinert, 1995). The properties of the four genes suggest that they might have a role as modulators or sensors of DNA lesions (reviewed in Lydall and Weinert, 1996). However, the biochemical activities of the corresponding proteins, their reciprocal interactions and their interactions with other factors are still under investigation.

We have observed that the effect of deleting the MEC3 gene can be partially suppressed by overexpressing DDC1, since sensitivity to MMS and HU and checkpoint defects of mec3Δ strains are diminished when Ddc1p is overproduced, indicating that high levels of Ddc1p can partially mimic Mec3p function. Several models can be envisaged to explain these results. For example, the two gene products might have partially overlapping functions. In this case, since MEC3 overexpression cannot suppress MMS sensitivity or checkpoint defects of ddc1Δ strains, overproduced Mec3p should require some limiting step(s) to be able to substitute for Ddc1p. Ddc1p and Mec3p might also perform subsequent functions, the Mec3p-dependent reaction preceding that involving Ddc1p. In this case, partial suppression of the mec3Δ phenotypes by DDC1 overexpression might result from a reduced requirement for upstream functions, including Mec3p. The observation that DDC1 overexpression also partially suppresses the sensitivity of rad9Δ mutants (our unpublished data) supports this ordering. Finally, phosphorylation of Ddc1p both during the normal cell cycle and in response to DNA damage is at least partially dependent on the presence of Mec3p. This finding not only provides insights into the relationships between MEC3 and DDC1, but also correlates Ddc1p phosphorylation with activation of DNA damage checkpoint pathways.

Taken together, our data indicate that Ddc1p participates together with Mec3p and, possibly, Rad17p and Rad24p in DNA damage recognition/processing events at an early step in the DNA damage response process, and it might be involved in the signal sensing and transducing branch of the pathway. Future work will be focused on understanding how this part of the pathway is integrated into the cascade of events leading to cell cycle arrest. To this end, it will be crucial to establish the functional role(s) of Ddc1p phosphorylation and its connections with the Mec1p and Rad53p general transducers, as well as to identify the kinase(s) responsible for Ddc1p modification.

**Materials and methods**

**Oligonucleotides used for PCR amplifications**

The following oligonucleotides were used: PRP33, 5’GGCTGATGTTCGCTACCGCTGTG3’; PRP34, 5’CGGGATCCATATGTCATTTAA-GGCTGATGTTAGACCCAGC3’; PRP46, 5’GGAATTCATATGTCATTTAAG-TAGGATGTCATTTAAG-GGCTGATGTTAGACCCAGCAGTCGAC3’; PRP49, 5’CTTAAGCATATGGGATCCTGCGCTTTTCTACTTGTGTTAGACCCAGC3’; PRP51, 5’GGCAACTATCACCGAGTGGGGCGTACGCTGCAGGTCGAC3’; PRP52, 5’TATACCCCTTGGCCTTTTCTACTTGTGTTAGACCCAGCAGTCGAC3’.

**Plasmids**

Plasmid PML80.1 is the original pUN100 derivative plasmid (Jansen et al., 1993), carrying a S.cerevisiae chromosome XVI fragment located...
Table 1. S. cerevisiae strains used in this study

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<td>MATa ade2-1 ade3 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100</td>
<td>this study</td>
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<td>YLL335</td>
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*aPlasmids are indicated within brackets*

between positions 175 452 and 186 891. Plasmid pML89 was obtained by replacing the XbaI–AccI fragment of plasmid YCplac111 (Gietz and Sugino, 1988) with the 2772 bp NsiI–BsBI DNA fragment, containing the DDC1 gene. To construct plasmid pML109, where the 2467 bp fragment, spanning from the DDC1 ATG to the Eco47III site and containing the whole DDC1 coding region, is fused to the GAL1 promoter, an EcoRI–BanHI fragment containing the GAL1–10 promoter was first used to replace the EcoRI–BanHI fragment within the YIp128 polylinker region (Gietz and Sugino, 1988), giving rise to plasmid pML95; a DDC1 fragment spanning from position +1 to position +173 from the translation initiation codon was then amplified by PCR using plasmid pML89 as a template and oligonucleotides PRP33 and PRP34 as primers and then cloned into the BanHI–HindIII sites of plasmid pML95, to give rise to plasmid pML101. The 4182 bp HindIII fragment from plasmid pML80.1 was then cloned into the HindIII site of plasmid pML101, followed by excision of the Eco47III–BglI DNA fragment from the DDC1 translation initiation codon to give rise to plasmid pML109. To construct plasmid pML118, carrying a 2HA-tagged GAL1–DDC1 fusion (GAL1–HA2–DDC1), plasmid B2358 (Kolodziej and Young, 1991) was used as a template for PCR amplification with oligonucleotides PRP49 and PRP46 as primers. The amplification product, containing two copies of the HA epitope-coding sequence, was cloned into the NdeI site at position +1716 from the DDC1 translation initiation codon of plasmid pML109, giving rise to plasmid pML118, whose 1664 bp XmaI–HindIII DDC1 fragment was then cloned into the Smal–HindIII sites of YIp1ac128, and the derivative plasmid pML119, carrying the 2HA-DDC1 allele, was used to construct strains YLL334 and YLL335 (see below). Plasmid pML1113, whose construction will be described elsewhere, is a YIp1ac128 derivative plasmid carrying the whole MEC3 coding sequence fused to the GAL1 promoter.

All the PCR reactions were carried out using Vent DNA polymerase (Biolabs). The fidelity of PCR amplification was controlled by nucleotide sequence analysis of the GAL1–DDC1 and GAL1–MEC3 fusions. Both the GAL1–DDC1 and the GAL1–MEC3 fusions were shown to complement the defects of the cognate null alleles. The chromosomal plasmids pK879 and pDL214, carrying the corresponding RAD17 and RAD24 genes, were a kind gift from D.Lydall (Tucson University, AZ). Plasmids pML78 and pML79 were constructed by cloning the RAD53 EcoRI–EcoRI fragment from plasmid pRS316-SPK1 (gift from D.Stern, Yale University, CT) and the MEC1 SpeI–SpeI fragment from plasmid pK9000 (gift from I.Ogawa, Osaka University), respectively, into the EcoRI and the SpeI sites of YCplac111.

Yeast strains and media

The genotypes of all the yeast strains used in this study are listed in Table 1. All the strains are derivatives of W303 (MATaMATa ade2-1 ade2-1 trp1-1/trp1-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 his3-11,15 ura3/ ura3). Strain YLL231 was derived from strain K2346CS33 by transformation with plasmid pML9 (Longhese et al, 1996a). Strain DMP1777/4D is a meiotic segregant from a cross of the original pps-1 mutant (Longhese et al., 1996a) with strain K2348CS33. Strain DMP1813/1A is a meiotic segregant from a cross between strains DMP1777/4D and K2348. One-step replacement of 1752 bp of the DDC1 coding region with the kanMX4 cassette (ddc1::kanMX4) was carried out by transforming strains K699, YLL231, YLL134 and YLL157 with a PCR-amplified kanMX4 cassette (see below) to give rise to strains YLL244, YLL245, YLL271 and YLL301, respectively. Strain DMP262/2C is a meiotic segregant from a W303 derivative heterozygous for the ddc1::kanMX4 allele (see below). Strains YLL280 and YLL288, carrying a single copy of a GAL1–DDC1 fusion integrated at the LEU2 locus, were obtained by transforming, respectively, strains K699 and YLL134 with BsrXI-digested plasmid pML109. Strains YLL302 and YLL303, carrying a single copy of a GAL1–MEC3 fusion integrated at the LEU2 locus, were obtained from strains K699 and YLL244, respectively, by transformation with BsrXI-digested plasmid pML113. Strains YLL334 and YLL335, carrying the 2HA-DDC1 allele at the DDC1 chromosomal locus, were obtained by transforming, respectively, strains K699 and YLL134 with PstI-digested plasmid pML119. The 2HA-DDC1 allele is fully functional, since strains K699 and YLL334 were indistinguishable from one another. The accuracy of all gene replacements and integrations was verified by Southern blot analysis. Standard yeast genetic techniques and media were according to Rose et al. (1990). YP media contained either 2% glucose (YPD), 2% galactose (YP-gal), 2% raffinose (YP-raffinose) or glucose (YPD), 2% galactose (YP-gal), 2% raffinose (YP-raffinose) or 5-FOA (Wach et al., 1994) in 400 μg/ml G418 (450 μg/ml, US Biological).

Cloning and disruption of the PIP5/DDC1 gene

To clone the gene identified by the pps-1 mutation, strain DMP1777/4D was transformed with a yeast genomic DNA library constructed in the pUN100 LEU2 centromeric plasmid (Jansen et al., 1993), and transformants were screened for the presence of recombinant plasmids able to restore a Scet* p-200 phenotype, and therefore possibly complementing synthetic lethality (Longhese et al., 1996a). Five different plasmids carrying partially overlapping yeast DNA inserts were identified by this screening. The minimal region complementing synthetic lethality was within an NsiI–BstI fragment (Figure 1A), and contained the PIPS5 gene, which we named DDC1 (see Results). To construct a DDC1 chromosomal deletion (ddc1::kanMX4) was amplified by PCR using plasmid pA6a-kanMX4 (Wach et al., 1994) as a template and oligonucleotides PRP21 and PRP22 as primers. The amplification product contained the kanMX4 cassette flanked by DDC1 sequences (underlined in the oligonucleotide sequences) and was used to transform the diploid strain W303. G418-resistant
transformants were shown by PCR analysis to be heterozygous for the replacement of most of the DDC1 chromosomal ORF with the kanMX cassette. By sporation and tetrad analysis of one of these transformants, ddc1Δa segregants were shown to be viable and to grow as wild-type on different media at different temperatures. A ddc1Δpip5-1 diploid strain obtained by crossing strain DMP262/2C to strain DMP1813/1A (see Table I) was as sensitive to UV, MMS and HU as the parent strains (data not shown). Although spore viability was severely affected, we could test 50 viable meiotic segregants from 30 tetrads of this diploid strain, and they were all sensitive to UV, MMS and HU, thus confirming that the ddc1Δ and pip5-1 mutations are allelic.

UV, MMS and HU synchrony experiments

Cell synchronization in G1 was obtained by treatment of exponentially growing YPD cell cultures with 2 μg/ml of α-factor, followed by release in YPD. G2 arrest was obtained by treating exponentially growing YPD cell cultures with 5 μg/ml of nocodazole and 1% dimethylsulfoxide (DMSO) until 90–95% of cells were large budded. α-Factor- and nocodazole-arrested cells were collected by centrifugation, and 5×10^6 cells were spread on 14 cm diameter YPD plates (Allen et al., 1994), followed by UV irradiation with 40 and 50 J/m², respectively. When required, cell cultures were held in G1 or G2 after UV irradiation by treatment with 2 μg/ml of α-factor or 15 of μg/ml nocodazole and 1% DMSO, respectively. MMS synchrony experiments were carried out as previously described (Paulovich and Hartwell, 1995) using respectively 0.02% MMS in YPD medium, and 0.015% MMS in galactose- and raffinose-containingYP medium. HU synchrony experiments were according to Allen et al. (1994), using 200 mM HU.

Protein extracts and Western blot analysis

Protein extracts for Western blot analysis were prepared from trichloroacetic acid-treated yeast cells as previously described (Foiani et al., 1994). Protein extracts were resolved by electrophoresis on 12.5% SDS–polyacrylamide gels and proteins were transferred to nitrocellulose membranes, which were then incubated for 2 h at room temperature with anti-HA monoclonal antibody 12CA5 (1:5000 dilution in Tris-buffered saline with 0.2% Triton X-100 and 4% non-fat milk), followed by incubation with peroxidase-labeled anti-mouse antibody (Amersham).

Immunoprecipitation and phosphatase treatment

Protein extracts for immunoprecipitation were prepared from exponentially growing cells collected by centrifugation and resuspended in an equal volume (v/v) of lysis buffer [0.1% SDS, 1% Triton X-100, 1% Na deoxycholate, 0.05 M Tris–HCl pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 20 μg/ml N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 60 μM β-glycerophosphate and 1 mM sodium orthovanadate]. After addition of 0.005 M Tris–HCl pH 7.8, 2 μM MnCl₂, 5 μM dithiothreitol, 100 μg/ml acetylated bovine serum albumin) and phosphatase treatment, the resin was resuspended in 20 μl of SDS–gel loading buffer and bound proteins were resolved by electrophoresis on a 12.5% SDS–polyacrylamide gel and visualized by Western blotting.

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References


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