

Two Distinct Pathways for Inhibiting Pds1 Ubiquitination in Response to DNA Damage*

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The presence of DNA damage activates a conserved cellular response known as the DNA damage checkpoint pathway. This pathway induces a cell cycle arrest that persists until the damage is repaired. Consequently, the failure to arrest in response to DNA damage is associated with genomic instability. In budding yeast, activation of the DNA damage checkpoint pathway leads to a mitotic cell cycle arrest. Following the detection of DNA damage, the checkpoint signal is transduced via the Mec1 kinase, which in turn activates two kinases, Rad53 and Chk1 that act in parallel pathways to bring about the cell cycle arrest. The downstream target of Rad53 is unknown. The target of Chk1 is Pds1, an inhibitor of anaphase initiation whose degradation is a prerequisite for mitotic progression. Pds1 degradation is dependent on its ubiquitination by the anaphase-promoting complex/cyclosome ubiquitin ligase, acting in conjunction with the Cdc20 protein (APC/C^{Cdc20}). Previous studies showed that the Rad53 and Chk1 pathways independently lead to Pds1 stabilization but the mechanism for this was unknown. In the present study we show that both the Chk1 and the Rad53 pathways inhibit the APC/C^{Cdc20}-dependent ubiquitination of Pds1 but they affect different steps of the process: the Rad53 pathway inhibits the Pds1-Cdc20 interaction whereas Chk1-dependent phosphorylation of Pds1 inhibits the ubiquitination reaction itself. Finally, we show that once the DNA damage is repaired, Pds1 dephosphorylation is involved in the recovery from the checkpoint induced cell cycle arrest.

The ability to sense and properly respond to the presence of DNA damage is crucial for cell viability, and DNA strand breaks that persist through mitosis can lead to the loss or gain of large chromosome fragments, resulting in aneuploidy. Cells have the ability to detect DNA damage and respond by activating an evolutionarily conserved mechanism known as the DNA damage checkpoint pathway (1). Consistent with its role in ensuring cellular integrity, defects in several checkpoint proteins enhance sensitivity to DNA-damaging agents and cause genomic instability (2). The checkpoint response leads to the transcriptional activation of numerous DNA repair genes; con-

comitantly, it induces a cell cycle arrest that provides the cell with a window of time during which DNA repair can take place. In higher eukaryotes, the DNA damage checkpoint pathway may induce apoptosis (3). Importantly, if cells are not led down the apoptotic route, the cell cycle will resume once the damage is repaired.

In most metazoans, one of the main arrest points of the DNA damage checkpoint pathway is in G₂, when entry into mitosis is prevented via the inactivation of the cyclin-dependent kinase Cdk1 (4). In budding yeast, however, the DNA damage checkpoint pathway does not control cell cycle progression by inactivating the Cdk1 equivalent, Cdc28 (5). Instead, this checkpoint pathway induces a mitotic arrest by inhibiting the metaphase to anaphase transition. This is accomplished, at least in part, by preventing the degradation of the mitotic regulator Pds1, as cells lacking Pds1 are unable to maintain a mitotic cell cycle arrest in the presence of DNA damage (6–10). Pds1 inhibits anaphase initiation by repressing the anaphase activator Esp1, a protease that dissolves sister chromatid cohesion (11, 12). Under normal conditions, the anaphase-promoting complex/cyclosome (APC/C)¹ promotes the ubiquitination of Pds1, leading to its degradation, and consequently the activation of Esp1 (13, 14). The ability of the APC/C to ubiquitinate Pds1 depends on the physical association between Pds1 and an APC/C-associated protein, Cdc20 (15–18). Specifically, abolishing the binding of Pds1 to Cdc20 prevents Pds1 ubiquitination (17, 18). Cdc20, and its homolog Cdh1, belong to a family of WD-repeat proteins that confer substrate specificity to the APC/C (15, 16). Cdc20 is active early in mitosis and is involved in the ubiquitination of several cell cycle regulators, including Pds1 and the cyclins Clb5 and Clb2 (19–21). Cdh1 becomes active in anaphase, only after Pds1 is degraded, and its activity is needed for the ubiquitination of APC/C substrates at the exit of mitosis and in G₁ (16, 19, 22).

The presence of DNA damage leads to the activation of the Mec1 kinase, which in turn activates two downstream kinases, Chk1 and Rad53 (Chk2 in mammalian cells). Chk1 and Rad53 are on two parallel branches of the DNA damage checkpoint pathway, and in budding yeast both are required for a complete metaphase cell cycle arrest (Fig. 1) (8, 9). The downstream target of Rad53 that is needed for the mitotic arrest is unknown. The target of Chk1 is Pds1, which is directly phosphorylated by Chk1 (9, 10). This phosphorylation is essential for Pds1 stabilization in the presence of DNA damage (10), but it is currently not known how this stabilization is achieved.

An important, yet poorly understood, aspect of the DNA damage checkpoint arrest is the recovery process. This is especially important for a unicellular organism such as budding yeast, where an arrest in response to a sublethal DNA damage

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¹ The abbreviations used are: APC/C, anaphase promoting complex/cyclosome; HA, hemagglutinin; IP, immunoprecipitation.

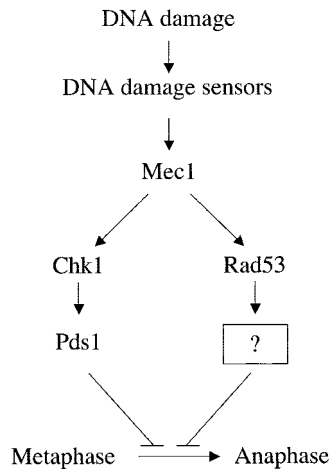


FIG. 1. The two parallel branches of the DNA damage checkpoint pathway. DNA damage is detected by numerous sensor proteins (not shown), and the signal is transduced to the Mec1 kinase. Mec1 activates two independent branches of the checkpoint pathway: one that is controlled by the Chk1 kinase and the other that is controlled by the Rad53 kinase. Both pathways are needed for a complete cell cycle arrest. Chk1 inhibits the metaphase to anaphase transition through the phosphorylation of Pds1 whereas the target of Rad53 is unknown.

dose without the possibility of recovery would be pointless. Only a few proteins involved in this recovery process have been identified thus far (Ref. 23 and references therein, Ref. 24), and little is known about the pathways that turn off the checkpoint signal and reactivate the cell cycle machinery once the DNA damage is repaired.

In this study we examined the mechanism by which the presence of DNA damage inhibits mitotic progression in budding yeast. We provide evidence that Pds1 ubiquitination is subjected to at least two inhibitory signals, one that is Rad53-dependent and the other that is Chk1-dependent. Our results also provide an insight into the mechanism by which cells recover from the DNA damage-induced checkpoint arrest.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—All the strains used in this work are W303 derivatives (*MATa bar1 ura3 leu2 trp1 his3 ade2*): OCF1522 (*PDS1-HA::URA3*) (7); ZH2601-3C (*CDC20-Myc::TRP1 cdc16-123*); ZH2601-1D, as ZH2601-3C but also *PDS1-HA::URA3* (18); RA2846-4A (*MET-CDC20::URA3 PDS1-HA::HIS3*); RA2850-21D (*CDC20-Myc::TRP1 mec1-1 cdc16-123*); RA2850-29B, as RA2850-21D but also *PDS1-HA::URA3*; RA2848 (*CDC20-Myc::TRP1 chk1Δ::kan cdc16-123*); RA2847, as RA2848 but also *PDS1-HA::URA3*; RA2864-8A (*CDC20-Myc::TRP1 cdc16-123 rad53-21*); RA2864-26C, as RA2864-8A but also *PDS1-HA::URA3*; JC-208 (*rad52ΔNAT PDS1-HA::URA3*). Strain 1120 (*PDS1-HA-LEU2::pds1, cdi1::HIS3*) was a generous gift from A. Amon (MIT Dept. of Biology, Cambridge, MA) Plasmid: pQL10 (*Gal-Chk1-HA URA3*) was a generous gift from Y. Sanchez, (University of Cincinnati, Cincinnati, OH).

Reagents— α -Mating pheromone (Sigma) was used at a concentration of 1 μ M. Nocodazole (Aldrich) was used at a concentration of 15 μ g/ml. Anti-HA antibodies (16B12) and protein A-Sepharose-linked antibodies were from Covance. For the methionine promoter shut-off experiments methionine was used at a concentration of 0.3 mg/ml.

Cell Lysis and Immunoprecipitation—Yeast cell lysates were prepared by breaking cells with glass beads in buffer A (0.05 M Tris-HCl at pH 7.5, 0.1 M NaCl, 5 mM EGTA, 5 mM EDTA, 0.025% Nonidet P-40, 10 mM sodium pyrophosphate, and protease inhibitors mix from Roche Applied Science). The extract (2 mg of protein) was incubated at 4 °C for 2 h with 40 μ l of anti-HA antibodies prebound to protein A-Sepharose. The immunoprecipitates were washed extensively in buffer A. Bound proteins were eluted by boiling in 50 μ l of 1 \times SDS sample buffer. Proteins were separated by SDS-PAGE (10% gel) and analyzed by Western blot analysis. Quantification of the amount of immunoprecipitation was done by densitometry scanning of the results from several independent experiments.

Protein Techniques—The preparation of protein extracts and the

purification of bacterially expressed Pds1 were as described (25). Separation of phosphorylated Pds1 on low cross-linking gels was as described (7).

Kinase Assays—Wild-type cells carrying Chk1-HA (pQL10) were grown to log phase at 30 °C in media containing raffinose, after which Chk1 expression was induced from a galactose-inducible promoter for 2 h. Protein extracts were prepared and Chk1-HA was immunoprecipitated using anti-HA antibodies as described above. For radioactive kinase assays, 2.5 μ g of purified recombinant Pds1 was mixed with 29 μ l of immunoprecipitated Chk1, 1 mM ATP, 50 μ g/ml creatine kinase, 35 mM phosphocreatine, 1.3 μ Ci/ μ l of [γ - 32 P]ATP, 5 mM MgCl₂ in buffer B (0.05 M Tris-HCl at pH 7.5, 0.1 M NaCl, 1.25 mM EGTA, 1.25 mM EDTA, 0.025% Nonidet P-40, and protease and phosphatase inhibitors) in a total volume of 40 μ l. As a control, purified recombinant Pds1 was incubated in the same buffer with the anti-HA immunoprecipitates from a strain that lacked the expression of Chk1-HA. The Chk1 bound or the control beads were removed by centrifugation and radiolabeled Pds1 was used *in vitro* ubiquitination reactions. The Pds1 protein treated with the control IP was not radiolabeled and used as the control in the ubiquitination assays.

In Vitro Ubiquitination Reaction—APC was purified from mitotic *Xenopus* egg extracts with the anti-Cdc27 (APC3) beads. The anti-Cdc27 beads were incubated with 10 volumes of mitotic *Xenopus* egg extracts for 2 h at 4 °C and washed five times with XB (10 mM HEPES, pH 7.7, 100 mM KCl, 0.1 mM CaCl₂, 1 mM MgCl₂, 50 mM sucrose) containing 500 mM KCl and 0.5% Nonidet P-40, and twice with XB. The mitotic APC beads were then incubated for 1 h at room temperature with purified recombinant human Cdc20 or Cdh1 proteins expressed in Sf9 insect cells. After incubation, the APC beads were washed twice with XB, and assayed for ubiquitination activity toward either unphosphorylated Pds1 or 32 P-labeled Pds1 protein phosphorylated by Chk1. Each ubiquitination assay was performed in a volume of 6 μ l at room temperature. The reaction mixture contained 1 mM ATP (Sigma), 150 μ M of bovine ubiquitin (Sigma), 5 μ M of human E1, 2 μ M of UbcH10, 3 μ l of the APC beads, and 1 μ l of unlabeled Pds1 treated with the control IP or 1 μ l of 32 P-labeled Pds1 modified by the Chk1 IP (see above). Both preparations of Pds1 had undergone the same treatment prior to the ubiquitination reactions. Samples were taken at the indicated time points, quenched with SDS sample buffer, and separated by SDS-PAGE. For the ubiquitination assays of the unlabeled Pds1 protein, the reaction mixtures were immunoblotted with the anti-Pds1 antibody. The intensities of the Pds1-ubiquitin conjugates were quantified with a densitometer. For the assays with the 32 P-labeled Pds1 protein, the reactions were analyzed and quantified with a Fuji phosphorimager. In both cases, the intensities of Pds1-ubiquitin conjugates were normalized against their molecular weights. The APC activity was determined as the total normalized intensity of the Pds1-ubiquitin conjugates.

RESULTS

The Presence of DNA Damage Blocks the Pds1-Cdc20 Interaction in a Rad53-dependent, Chk1-independent Manner—In budding yeast, the presence of DNA damage activates the DNA damage checkpoint pathway, which stabilizes Pds1, thereby blocking mitotic progression. At least part of this stabilization can be attributed to the phosphorylation of Pds1 by Chk1 (9, 10). We thus set out to determine how Chk1 phosphorylation inhibits the degradation of Pds1. The electrophoretic mobility of Pds1 extracted from cells that have experienced DNA damage showed that under these conditions Pds1 was phosphorylated, and it did not accumulate as polyubiquitinated forms (7, 9). This indicated that the stabilization of Pds1 was not due to the inhibition of degradation *per se* but due to a block at, or prior to, ubiquitination. We have previously shown that Pds1 ubiquitination by its ubiquitin ligase, the anaphase-promoting complex/cyclosome (APC/C) acting in conjunction with Cdc20, depended on the direct interaction between Pds1 and Cdc20 (18). To test if the presence of DNA damage stabilizes Pds1 by affecting the Pds1-Cdc20 interaction, we examined whether Pds1 and Cdc20 can be co-immunoprecipitated from protein extracts prepared from DNA damaged cells. DNA damage was induced by exposing cells to γ radiation that created DNA double-strand breaks. In budding yeast the repair of these lesions largely depends on recombinational repair, and thus irradiation of haploid cells in G₁, lacking both homologous

chromosomes and sister chromatids, generates irreparable DNA damage (26, 27). Once these cells are released from the G₁ arrest, this persistent DNA damage leads to a DNA damage checkpoint-dependent metaphase arrest. Since the non-irradiated control cells would not arrest in metaphase, the strains used in this experiment carried a *cdc16* mutant gene that at the non-permissive temperature causes a metaphase arrest due to the inactivation of the Cdc16 subunit of the APC/C. Moreover, because the Pds1-Cdc20 interaction results in the rapid degradation of Pds1, the *cdc16* mutation enabled us to capture this transient interaction (18).

To examine whether DNA damage affected the Pds1-Cdc20 interaction we constructed *cdc16* mutant strains expressing Myc-tagged Cdc20 and either HA-tagged Pds1 or untagged Pds1 that served as a control. Cells were arrested in G₁ with α -mating pheromone, irradiated, and then released from the G₁ arrest by removing the pheromone and shifting the cells to 37 °C, the non-permissive temperature for *cdc16* mutant strains. Once cells reached the metaphase arrest, the ability of Pds1-HA to interact with Cdc20-Myc was examined by co-immunoprecipitation using anti-HA antibodies (Fig. 2A). As shown previously, in non-irradiated cells, Cdc20-Myc co-immunoprecipitated with Pds1-HA (Fig. 2A, lane 5) but not with an untagged Pds1 control (Fig. 2A, lane 7). In contrast, the presence of DNA damage markedly reduced the interaction between Cdc20-Myc and Pds1-HA (Fig. 2A, lane 6 and Fig. 2E). Given that the Pds1-Cdc20 interaction is essential for the ubiquitination of Pds1 (18), inhibiting this interaction would lead to Pds1 stabilization.

To confirm that the DNA damage-dependent inhibition of the Pds1-Cdc20 interaction was mediated by the DNA damage checkpoint pathway, we repeated the co-immunoprecipitation experiment using cells that were defective in the checkpoint response due to a *mec1* mutation. In this strain, in the presence of DNA damage Pds1 is neither phosphorylated nor stabilized (7, 9). In *mec1* mutant cells the presence of DNA damage did not block the Pds1-Cdc20 interaction (Fig. 2B, lanes 5 and 6, and Fig. 2E). The difference in the fraction of Cdc20 that co-immunoprecipitated with Pds1-HA in irradiated wild-type versus irradiated *mec1* strains (21.8 ± 8.8% and 80.5 ± 12% of the amount that co-precipitated in non-irradiated cells, respectively) was statistically significant, as determined by unpaired Student's *t* test ($p = 0.0307$) (Fig. 2E). Thus we conclude that the Pds1-Cdc20 interaction is subjected to regulation by the DNA damage checkpoint pathway.

Since Pds1 undergoes Chk1-dependent phosphorylation, we next examined whether the inability of Pds1 and Cdc20 to interact in the presence of DNA damage was due to Pds1 phosphorylation by Chk1. To address this question, the immunoprecipitation experiments were done with extracts from irradiated and non-irradiated cells lacking Chk1 function. As seen in Figs. 2C (lanes 5 and 6) and 2E, the Pds1-Cdc20 interaction was still blocked in a *chk1*Δ mutant. The difference in the amounts of Cdc20 co-precipitating with Pds1-HA in DNA damaged wild-type versus *chk1*Δ cells (21.8 ± 8.8% and 6.5 ± 8.6% of the amount that co-precipitated in non-irradiated cells, respectively) was insignificant ($p = 0.2207$). This suggested that a DNA damage checkpoint function other than Chk1-mediated phosphorylation is regulating the Pds1-Cdc20 interaction.

Given that Rad53 is also responsible for the stabilization of Pds1, we examined the Cdc20-Pds1 interaction in cells defective for Rad53 checkpoint function. In this strain Pds1 is still phosphorylated by Chk1 in response to DNA damage (7, 9). Interestingly, in the absence of Rad53 checkpoint function, the presence of DNA damage no longer abolished the Cdc20-

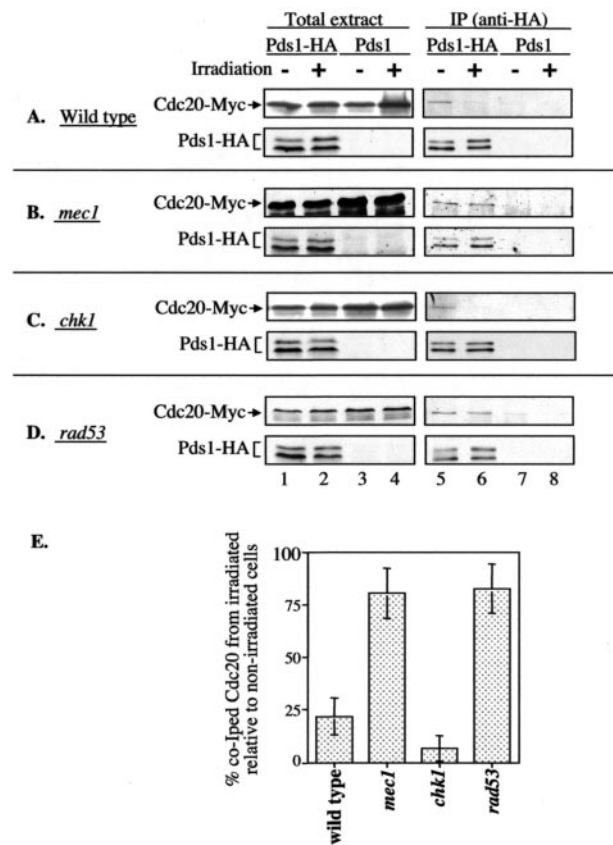


FIG. 2. The presence of DNA damage inhibits the Pds1-Cdc20 interaction in vivo. A, *cdc16* mutant strains expressing Cdc20-Myc and HA-tagged Pds1 (ZH2601-1D) or untagged Pds1 (ZH2601-3C) were grown at 23 °C, arrested in G₁ with α -factor and either irradiated (γ radiation, 8 krad) or left untreated. Cells were then released from the G₁ arrest at 37 °C to inactivate the *cdc16* gene product. Cell extracts were prepared as described under "Experimental Procedures" and subjected to immunoprecipitation using anti-HA antibodies coupled to protein A-Sepharose beads. Pds1-HA and Cdc20-Myc were detected by Western blot analysis using anti-HA and anti-Myc antibodies, respectively. Lanes 1–4, total extracts; lanes 5–8, the products of the immunoprecipitation reactions. B–D, same immunoprecipitation procedure as described in A, except that *mec1*, *chk1*, or *rad53* mutant strains, respectively, were used. E, quantification of the relative Cdc20 levels that co-immunoprecipitated with Pds1 in the absence and presence of DNA damage. The relative levels of precipitating Cdc20-Myc were quantified by densitometry scan of several independent experiments for each strain, calculating the relative amounts of Cdc20-Myc the co-immunoprecipitated with Pds1-HA. These values were normalized to the total amount of Cdc20-Myc present in the starting extracts (left panels), and the results are shown as the percent of co-immunoprecipitating Cdc20-Myc in the presence of DNA damage out of the amount of Cdc20 that co-precipitated with Pds1-HA in the same strain but in the absence of DNA damage (e.g. a 25% value indicates that the amount of Cdc20-Myc that co-immunoprecipitated with Pds1-HA in the presence of DNA damage is 4-fold lower than the amount of Cdc20-Myc that co-immunoprecipitated with Pds1-HA in the same strain, but in the absence of DNA damage).

Pds1 interaction (Fig. 2D, lanes 5 and 6, and Fig. 2E). We find that the difference in the amounts of Cdc20 co-precipitating with Pds1-HA in DNA damaged wild-type versus *rad53* mutant cells (84 ± 12.7% of the amount that co-precipitated in non-irradiated *rad53* mutant cells) was significant ($p = 0.0295$), whereas the amounts of Cdc20 associated with Pds1 in DNA-damaged *mec1* and *rad53* mutant strains were not significantly different ($p = 0.8036$). Thus, it is the Rad53 branch of the DNA damage checkpoint pathway that stabilizes Pds1 by inhibiting its interaction with Cdc20.

Chk1 Phosphorylation Is Sufficient to Inhibit the Ubiquitination of Pds1 by APC/C^{Cdc20}—Our results suggest that mul-

multiple inputs regulate the Pds1-Cdc20 interaction: Rad53 controls the Pds1-Cdc20 interaction whereas Chk1 phosphorylation must affect the stability of Pds1 through a different mechanism. To examine the effect of Chk1 phosphorylation *per se*, we tested whether Pds1 phosphorylated by Chk1 can undergo APC/C^{Cdc20}-dependent ubiquitination. We imagine that *in vivo*, one of two possible scenarios may take place. 1) Phosphorylated Pds1 may be a poor substrate for APC/C^{Cdc20}. 2) Phosphorylated Pds1 is a fine APC/C^{Cdc20} substrate but *in vivo* phosphorylated Pds1 associates with another protein(s) that either blocks its accessibility to APC/C^{Cdc20} or stimulates a deubiquitination reaction. The difference between the two scenarios is that in the former phosphorylation is sufficient to stabilize Pds1 whereas in the latter phosphorylation is not sufficient, and stabilization is achieved through the interaction of phosphorylated Pds1 with another protein(s). To test whether Chk1 phosphorylation is sufficient to block Pds1 ubiquitination we utilized an *in vitro* APC/C ubiquitination system that included purified *Xenopus* APC/C (28) with either recombinant Cdc20 or recombinant Cdh1. As substrate we used recombinant Pds1, with and without Chk1 phosphorylation. Although *in vivo* Pds1 is only subjected to ubiquitination by APC/C^{Cdc20}, in the *in vitro* reaction recombinant Pds1 was ubiquitinated by either APC/C^{Cdc20} or APC/C^{Cdh1} (Fig. 3A), probably because under these conditions Cdh1 is not subjected to the same inhibitory phosphorylation that it experiences *in vivo* (see below). To test whether Chk1 phosphorylation was sufficient to block the ubiquitination of Pds1, Pds1 was phosphorylated *in vitro* by immuno-purified Chk1 and subjected to ubiquitination by either APC/C^{Cdc20} or APC/C^{Cdh1}. To follow the fate of the Pds1 that underwent phosphorylation by Chk1, the phosphorylation reaction included [³²P]ATP, thus allowing us to specifically detect phosphorylated Pds1 by autoradiography. As shown in Fig. 3, panels B–D, phosphorylation of Pds1 by Chk1 substantially reduced the ubiquitination of Pds1 by APC/C^{Cdc20}. The difference in ubiquitination of Pds1 with and without Chk1 phosphorylation was statistically significant, as determined by the unpaired *t* test ($p = 0.0018$ for the 30 min time point and $p = 0.0149$ for the 60 min time point). In contrast, Chk1-phosphorylated Pds1 was ubiquitinated by APC/C^{Cdh1} as efficiently as unphosphorylated Pds1 (Fig. 3, panels B–D, the differences in ubiquitination were found to be not significantly different ($p = 0.9702$ for the 60 min time point)). The APC/C^{Cdh1} result shows that Chk1 phosphorylation did not have a general adverse affect on the ubiquitination reaction. Rather, Chk1 phosphorylation specifically inhibited APC/C^{Cdc20} from ubiquitinating Pds1. Therefore, Chk1-mediated phosphorylation is sufficient to stabilize Pds1 by disrupting the ability of Pds1 to be ubiquitinated by APC/C^{Cdc20}. Taken together our results suggest that in response to DNA damage Pds1 ubiquitination is inhibited at two different levels and by two independent branches of the DNA damage checkpoint pathway. The Rad53 pathway inhibits the Cdc20-Pds1 interaction whereas Chk1 interferes with the ability of APC/C^{Cdc20} to use Pds1 as a substrate.

Cdc20 Activity Is Needed for Recovery from the DNA Damage-induced Checkpoint Arrest—In unicellular organisms, the cell cycle arrest induced by the DNA damage checkpoint pathway is mostly useful if cells can recover and resume cell cycle progression after the damage is repaired. Little is known about the recovery process, how the checkpoint signals are turned off and how the inhibition by phosphorylated Pds1 is overcome. Cell cycle progression necessitates the degradation of Pds1. Because, as shown above, phosphorylated Pds1 is not a substrate for APC/C^{Cdc20}, recovery could involve the dephosphorylation of Pds1. Alternatively, our *in vitro* results show that at

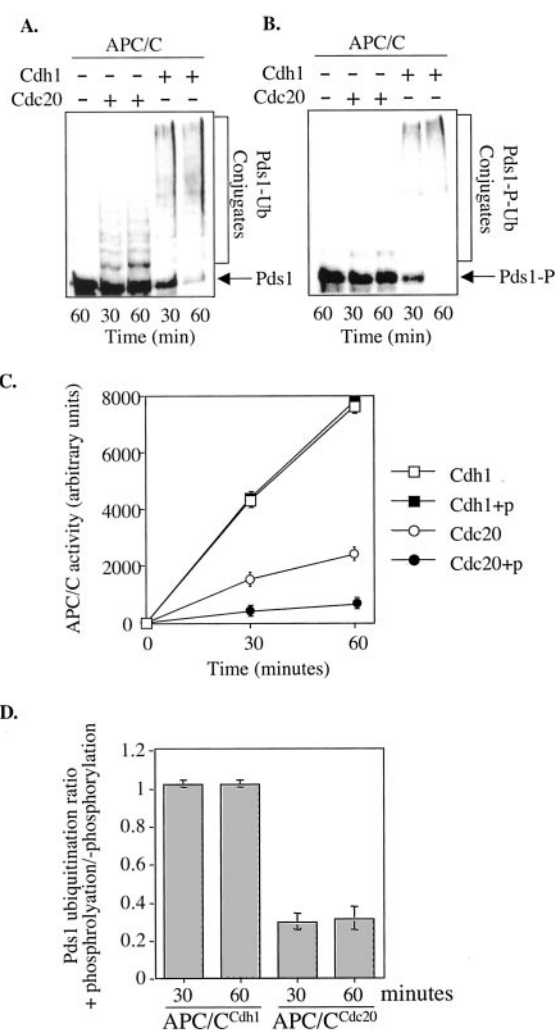


FIG. 3. Pds1 phosphorylation by Chk1 is sufficient to prevent its ubiquitination by APC/C^{Cdc20}. *In vitro* ubiquitination reactions of purified Pds1 (panel A) and Chk1-phosphorylated Pds1 (radiolabeled by ³²P) (panel B), using purified *Xenopus* APC/C with either Cdc20 or Cdh1, were carried out as described under "Experimental Procedures." Reaction products were resolved by 10% SDS-PAGE. Pds1 was detected using anti-Pds1 antibodies (panel A) and radiolabeled Pds1 was detected by autoradiography (panel B). The relative amounts of ubiquitinated proteins from several experiments as shown in panels A and B are plotted in panel C. Panel D shows the fraction of Pds1 that is ubiquitinated by APC/C^{Cdc20} or APC/C^{Cdh1} when Pds1 is phosphorylated by Chk1, as compared with Pds1 levels of ubiquitination in the absence of phosphorylation.

least in theory APC/C^{Cdh1} can ubiquitinate phosphorylated Pds1, raising the possibility that recovery may be associated with an APC/C^{Cdh1}-dependent degradation of Pds1. *In vivo*, during normal cell cycles, at the time when Pds1 is degraded APC/C^{Cdh1} is inactive due to inhibitory phosphorylation of Cdh1 by Cdc28/C1b (16, 19). This inhibitory phosphorylation is removed only after cells initiate anaphase, when very little Pds1 is still present in the cell. However, it was formally possible that during the recovery process APC/C^{Cdh1} is somehow activated to promote the degradation of phosphorylated Pds1. Had this been the case, cells lacking Cdh1 would be unable to recover from a DNA damage-induced checkpoint arrest once the damage is repaired. To test this possibility, DNA damage was induced under conditions that allowed for DNA repair to take place. This was achieved by irradiating cells arrested in metaphase with nocodazole, a spindle-depolymerizing drug that causes a metaphase arrest through the activation of the spindle assembly checkpoint. Irradiation of

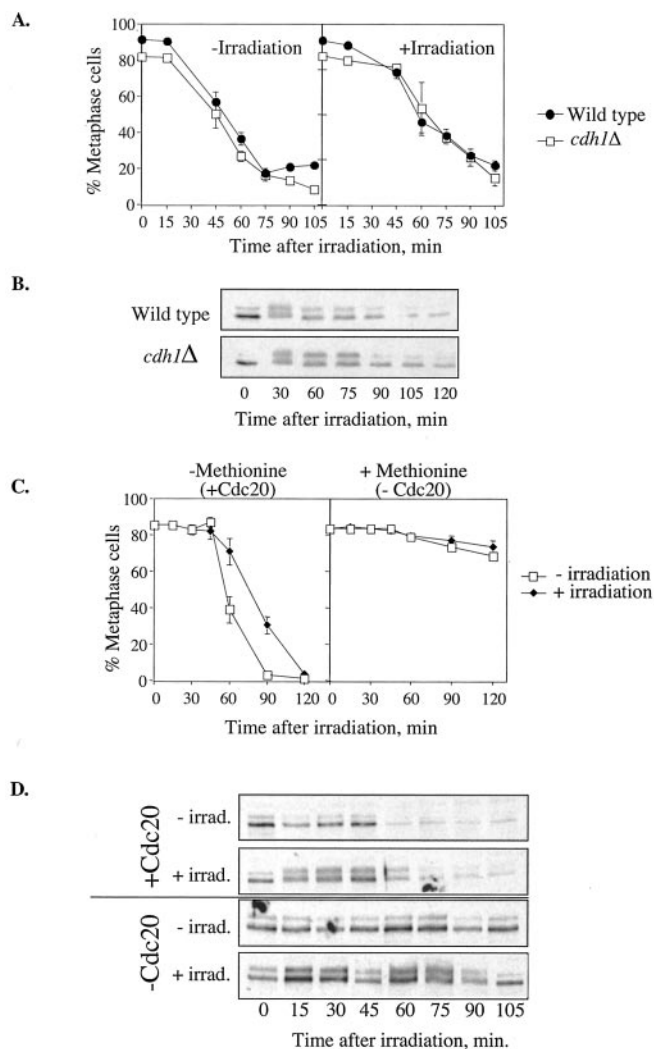


FIG. 4. Cdc20, but not Cdh1, is required for the recovery from a DNA damage-induced cell cycle arrest. *A*, wild-type cells (OCF1522, black circles) and *cdh1Δ* cells (1120, white squares) expressing Pds1-HA were grown at 30 °C, arrested in metaphase with nocodazole and either left untreated (*panel A, left*) or irradiated (γ radiation, 8 krad, *right*). Cells were released from the nocodazole arrest, samples were collected every 15 min and tested for cell morphology. The percentage of large budded cells with a single nucleus (indicative of metaphase cells) is shown. Time points where the error bars are not visible have a S.D. of 2.8% or less. *B*, Pds1-HA levels in irradiated wild-type and *cdh1Δ* cells as described in *panel A*. *C* and *D*, cells expressing CDC20 from a methionine-responsive promoter (RA2846-4A) were arrested in metaphase with nocodazole and either irradiated (γ radiation, 12 krad, black diamonds) or left untreated (white squares). The two cultures were then split and released into media without methionine (*left panel*) thereby allowing CDC20 expression, or with methionine (*right panel*), conditions that repress CDC20 expression. The media also contained α factor to prevent the cells from initiating another round of cell division once they reach G₁. *Panel C* shows the fraction of metaphase cells under each of the conditions described above (error bars of 2.8% S.D. or less are not visible), whereas *panel D* shows the levels of Pds1-HA under each of these conditions.

cells at this phase of the cell cycle does not lead to a permanent DNA damage checkpoint-induced cell cycle arrest because sister chromatids can serve as partners in recombinational repair (26, 27). To examine whether Cdh1 is required for post-repair recovery, wild-type and *cdh1Δ* mutant cells were arrested in metaphase with nocodazole, irradiated or left untreated and then released from the nocodazole block. With non-irradiated wild-type and *cdh1Δ* cells, the fraction of the metaphase-arrested cells was reduced by almost 50% within 45 min following irradiation (Fig. 4A, left panel). The exposure of wild-type cells

to DNA damage resulted in a delay in progression through mitosis, probably due to the time needed to repair the DNA damage and to inactivate the DNA damage checkpoint pathway. Nonetheless, 60 min following irradiation, over half the cells had left metaphase arrest (Fig. 4A, right panel). By 90 min after irradiation the vast majority of cells recovered from the metaphase arrest and most of Pds1 was degraded (Fig. 4B). Importantly, *cdh1Δ* cells recovered from the DNA damage-induced arrest in a manner that was indistinguishable from that of wild-type cells, both in terms of cell morphology (Fig. 4A, right panel) and kinetics of Pds1 degradation (Fig. 4B). Thus Cdh1 is not required for recovery from a DNA damage-induced arrest.

What, then, directs the degradation of Pds1 during the recovery from the DNA damage-induced arrest? APC/C^{Cdc20} is active in mitosis and is necessary for Pds1 ubiquitination and degradation under normal growth conditions. In fact, Pds1 is the only APC/C^{Cdc20} substrate that must be degraded in order to progress from metaphase to anaphase (6, 20). In other words, if, in the presence of DNA damage, Pds1 was ubiquitinated or degraded in a Cdc20-independent manner, Cdc20 would become dispensable for anaphase initiation. Conversely, a requirement for Cdc20 in initiating anaphase following the repair of DNA damage strongly suggests that Pds1 must be ubiquitinated by APC/C^{Cdc20} in order for the cells to recover from the cell cycle arrest. To test directly whether Cdc20 was needed for the recovery process, the *CDC20* gene was placed under a methionine-sensitive promoter such that expression was repressed in the presence of methionine. Cells were irradiated under conditions where the damage can be repaired, as described above, and the ability of cells to recover was examined in the presence or absence of Cdc20 activity (Fig. 4, panels C and D). In the presence of Cdc20 cells were able to recover from the DNA damage-induced arrest and the irradiated cells exited metaphase with a delay comparable to that observed previously (Fig. 4C, left panel). Pds1 levels were also reduced as cells exited metaphase (Fig. 4D, top panels). On the other hand, no metaphase exit was seen when the expression of *CDC20* was repressed, either in the presence or absence of DNA damage (Fig. 4C, right panel), and under both conditions Pds1 remained stable (Fig. 4D, lower panels). Had the DNA damage checkpoint pathway induced a Cdc20-independent mechanism for Pds1 ubiquitination, the cells would have escaped the metaphase arrest, despite the absence of Cdc20 activity. The fact that when Cdc20 was absent cells remained arrested after the DNA damage was repaired suggests that Cdc20 is needed for the recovery from a DNA damage-induced checkpoint arrest.

Pds1 Is Dephosphorylated following DNA Damage Repair—Two conditions must be met in order for Pds1 to be ubiquitinated following the repair of DNA damage: Pds1 must associate with Cdc20 and it must be ubiquitinated by APC/C^{Cdc20}. At this point we do not know how Rad53 abolishes the Cdc20-Pds1 interaction, but we do know that Chk1 inhibits APC/C-dependent ubiquitination by phosphorylating Pds1. Given that APC/C^{Cdc20} cannot efficiently ubiquitinate Chk1-phosphorylated Pds1 (Fig. 3), and since Cdc20 is needed to recover from the DNA damage-induced arrest (Fig. 4C), we considered the possibility that Pds1 undergoes dephosphorylation concomitant with recovery. The phosphorylation status of Pds1 can be monitored by SDS-PAGE (7): in the absence of DNA damage (Figs. 4B and 5A, time 0) Pds1 appears as a doublet, with the fast migrating band being of greater intensity than the slow migrating band. The slower migrating form of Pds1 is constitutive and is a result of Cdc28-dependent phosphorylation that does not change upon DNA damage (25). In the presence of DNA damage (Figs. 4B and 5A, most noticeable at 15 min post-

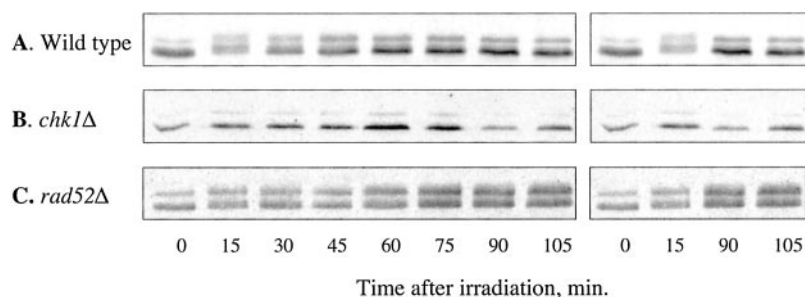


FIG. 5. **Pds1 is dephosphorylated following DNA damage repair.** A, wild-type cells expressing Pds1-HA (OCF1522) were grown in YPD at 23 °C, arrested in metaphase with nocodazole and exposed 8 krad of γ -radiation. Cells were then washed with fresh media + nocodazole, samples were taken every 15 min, and protein extracts were prepared and analyzed by Western blot analyses using anti-HA antibodies (*left panel*). The extracts taken at time points 0, 15, 90, and 105 min were analyzed next to each other to facilitate a direct comparison between the early and late time points (*right panel*). B and C, same as in *panel A*, except that the experiment was done using a *chk1Δ* strain in which Pds1 does not undergo a DNA damage-dependent phosphorylation (*panel B*), or a *rad52Δ* strain that is defective in homologous recombination, and as a result most of the DNA damage cannot be repaired (*panel C*).

irradiation) both bands shift up slightly and the intensities of the two bands becomes equivalent. This DNA damage-induced change in mobility does not appear in the absence of Chk1 activity (Fig. 5B, the 15 min time point). To determine if Pds1 is dephosphorylated prior to its degradation as the DNA damage was being repaired (60–90 min following irradiation, Fig. 4A), cells were arrested in metaphase with nocodazole, irradiated and kept in metaphase with nocodazole throughout the duration of the experiment. This allowed us to monitor the phosphorylation status of Pds1 without the complication that Pds1 is degraded as cells leave the metaphase arrest. If Pds1 is dephosphorylated following DNA damage repair, we should observe the conversion of the Chk1-phosphorylated Pds1 (*e.g.* 15 min post-irradiation) to the pre-irradiation, constitutive Pds1 forms at around 90 min post-irradiation. As seen in Fig. 5A, Pds1 was gradually dephosphorylated, and after 90 min the Chk1 phosphorylated forms were no longer detected. To visualize this difference more clearly, protein samples from time points 0, 15, 90, and 105 min post-irradiation were analyzed side-by-side (Fig. 5A, *right panel*). These results suggest that Pds1 is dephosphorylated following the repair of DNA damage, concomitant with the recovery from the cell cycle arrest. Consistent with this possibility, when DNA damage was induced in cells that were unable to repair double strand breaks, such as in a *rad52* mutant strain that is defective for homologous recombination, Pds1 became phosphorylated following the introduction of DNA damage, and this phosphorylation was maintained throughout the duration of the experiment, long after the phosphorylated Pds1 forms disappeared in the irradiated wild-type strain (compare Fig. 5, C (*rad52Δ*) and A (wild-type)). Taken together, these results suggest that Pds1 is dephosphorylated following the repair of DNA damage.

DISCUSSION

This study was aimed at understanding the mechanism underlying the stabilization of Pds1 in the presence of DNA damage. *In vivo*, in order to be ubiquitinated, Pds1 must bind Cdc20 (18). In examining the effect of DNA damage on the ability of Pds1 to interact with Cdc20 we uncovered a previously unknown mechanism for stabilizing Pds1, through the inhibition of the Pds1-Cdc20 interaction. This mechanism is Mec1-dependent, Rad53-dependent but Chk1-independent. Thus, our findings strongly suggest that at least one of the previously unknown targets of the Rad53 branch is the Pds1-Cdc20 interaction. We propose that Rad53 inhibits anaphase initiation, either directly or indirectly, by inhibiting the ability of Pds1 to associate with Cdc20 (Fig. 6). At this point we do not know how Rad53 regulated the Pds1-Cdc20 interaction. Neither Pds1 nor

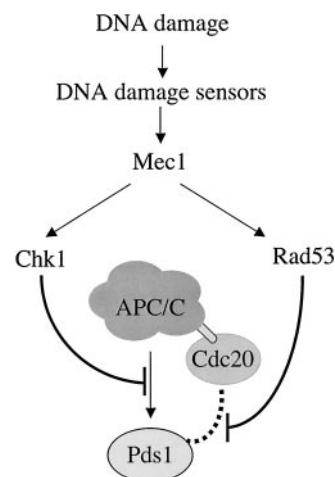


FIG. 6. **The ubiquitination of Pds1 is subjected to regulation by the two branches of the DNA damage checkpoint pathway.** Results presented in this study suggest that Rad53 inhibits the ability of Pds1 and Cdc20 to interact, whereas Chk1 inhibits the ubiquitination reaction itself through the phosphorylation of Pds1. See text for more details.

Cdc20 change their localization in response to DNA damage.² It is thus tempting to speculate that the Pds1-Cdc20 interaction may be inhibited by modification of the Pds1 or Cdc20 proteins, or by the presence of one or more proteins that prevent Pds1 from binding to Cdc20. These possibilities are currently under investigation.

Chk1 phosphorylation inhibits Pds1 ubiquitination but the exact mechanism remains unknown. Our *in vitro* results show that Chk1-dependent phosphorylation of Pds1 is sufficient to inhibit the ubiquitination of Pds1 by APC/C^{Cdc20} (Fig. 3), suggesting that phosphorylation directly blocks APC/C^{Cdc20} from using Pds1 as a substrate (Fig. 6). A plausible explanation is that the phosphorylation of Pds1 by Chk1, which occurs on at least 9 different sites (10) renders Pds1 a poor APC/C^{Cdc20} substrate. Taken together, our findings suggest that two separate DNA damage checkpoint mechanisms affect Pds1 stability: one that is Chk1-dependent and that affects the ability of the APC/C to use Pds1 as a substrate due to Pds1 phosphorylation, and the other that is Rad53-dependent and that affects the Pds1-Cdc20 interaction (Fig. 6). This conclusion is consistent with the partial cell cycle arrest observed in *chk1Δ* and *rad53Δ* mutant strains in response to DNA damage (8, 9). Rad53 is likely to have additional targets besides Pds1, because

² K. Ross and O. Cohen-Fix, unpublished observations.

pds1Δ strains still show a partial cell cycle arrest in response to DNA damage, and this arrest is Rad53-dependent. The nature of this other Rad53 target remains unknown.

Our results also explain a previous observation in which overexpression of Cdc20 could override a DNA damage checkpoint arrest (29). In light of our results it is conceivable that exceedingly high levels of Cdc20 can overcome the inhibitory signals imposed by the DNA damage checkpoint pathway. Interestingly, the mechanism by which Pds1 is stabilized in the presence of DNA damage is similar to the one described for p53, which is phosphorylated in response to DNA damage by a different checkpoint kinase, Chk2 (for review see Ref. 30). Under normal conditions p53 is destabilized through its association with the MDM2 ubiquitin ligase, and recent studies show that p53 phosphorylation disrupts the MDM2-p53 interaction, thereby stabilizing p53. Thus, achieving protein stability by disrupting the ubiquitin ligase-substrate interaction through substrate phosphorylation appears to be a universal mechanism.

An important question is how Pds1 is made accessible to degradation once the DNA damage is repaired. It is unlikely that *in vivo* Cdh1 plays a role in Pds1 degradation because (a) at metaphase Cdh1 is inhibited from binding the APC/C due to Cdc28-Clb phosphorylation (16, 19, 31, 32), and (b) our results show that Cdh1 is dispensable during the recovery process (Fig. 4, A and B). It is also unlikely that Pds1 is degraded through an APC/C-independent mechanism because Pds1 levels remain high when the APC/C itself is inactive (Ref. 13 and data not shown). We show here that Cdc20 is needed for the recovery process (Fig. 4C). Given our observation that APC/C^{Cdc20} cannot ubiquitinate phosphorylated Pds1, we speculated that Pds1 is dephosphorylated as the DNA damage is repaired. Consistent with this possibility, we observed that under conditions where the DNA damage could be repaired Pds1 was indeed dephosphorylated, and this coincided with the time at which cells recovered from the metaphase arrest. Therefore, we propose that Pds1 phosphorylation by Chk1 leads to a cell cycle arrest during which the DNA damage is repaired. Once the damage is removed, the Rad53 inhibitory signal is reversed and Pds1 undergoes dephosphorylation, making it accessible again to the ubiquitination machinery. The nature of the phosphatase(s) involved in the Pds1 dephosphorylation process is currently under investigation. Studies by Marsolier *et al.* (33) suggested a link between PP2C-type phosphatases and adaptation to, or recovery from, DNA damage. More recently, Leroy *et al.* (24) showed that the Ptc2 and Ptc3 phosphatases are involved in the dephosphorylation of Rad53 during the recovery from a cell cycle arrest caused by an HO-induced DNA double strand break. Our preliminary data do not show an involvement of Ptc2 and Ptc3 in Pds1 dephosphorylation,³ but it

should be noted that the type of damage examined in the Leroy *et al.* study, and as a result the time scales following the arrest that phosphatase activity was needed, are different from our experimental system. It is also not known whether phosphatase activity is induced following DNA damage repair, or whether it is constitutive, eventually overcoming protein phosphorylation once the checkpoint kinases turned off. It will be interesting to determine how phosphatases contribute to the recovery from the DNA damage-induced cell cycle arrest.

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³ R. Agarwal and O. Cohen-Fix, unpublished observations