Pds1p, an Inhibitor of Anaphase in Budding Yeast, Plays a Critical Role in the APC and Checkpoint Pathway(s)

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Abstract. We report the isolation and characterization of *pds1* mutants in *Saccharomyces cerevisiae*. The initial *pds1-1* allele was identified by its inviability after transient exposure to microtubule inhibitors and its precocious dissociation of sister chromatids in the presence of these microtubule inhibitors. These findings suggest that *pds1* mutants might be defective in anaphase arrest that normally is imposed by a spindle-damage checkpoint. To further examine a role for Pds1p in anaphase arrest, we compared the cell cycle arrest of *pds1* mutants and *PDS1* cells after: (*a*) the inactivation of Cdc16p or Cdc23p, two proteins that are required for the degradation of mitotic cyclins and are putative components of the yeast anaphase promoting complex (APC); (*b*) the inactivation of Cdc20p, another protein implicated in the degradation of mitotic cyclins; and (*c*) the inactivation of Cdc13 protein or γ irradiation, two circumstances that induce a DNA-damage checkpoint. Under all these conditions, anaphase is inhibited in *PDS1* cells but not in *pds1* mutants. From these results we suggest that Pds1 protein is an anaphase inhibitor that plays a critical role in the control of anaphase by both APC and checkpoints. We also show that *pds1* mutants exit mitosis and initiate new rounds of cell division after γ irradiation and Cdc13p inactivation but not after nocodazole-treatment or inactivation of Cdc16p, Cdc20p or Cdc23p function. Therefore, in the DNA-damage checkpoint, Pds1p is required for the inhibition of cytokinesis and DNA replication as well as anaphase. The role of Pds1 protein in anaphase inhibition and general cell cycle regulation is discussed.

ANAPHASE is the stage of mitosis in which replicated chromosomes (sister chromatids) segregate from one another. Before anaphase sister chromatids are paired along their lengths and condensed. Each pair of sister chromatids achieve a bipolar attachment to the spindle by binding to microtubules emanating from each spindle pole. At the onset of anaphase, the cohesion responsible for the sister chromatid association is dissolved, and the sister chromatids separate. Subsequently, sister chromatids segregate from one another by their movement towards opposite spindle poles and by the elongation of the spindle. These profound changes in the spindle and sister chromatid pairing at the onset of anaphase are regulated tightly; the mechanism of this regulation has been intensively studied over the past few years.

One important aspect of anaphase regulation is its timing relative to other cell cycle events. For example, anaphase must initiate before cytokinesis but not before S phase. The timing of anaphase during the cell cycle is mediated by p34 kinase and cyclins, a conserved family of activators (reviewed in Murray, 1991; Nigg, 1993). When a specific subset of cyclins associate with p34 kinase, a cascade of cell cycle events occur that are prerequisites for anaphase (reviewed in Nurse, 1990). Initially it was thought that the subsequent degradation of these cyclins destroyed p34 kinase activity and drove the cell into anaphase (Murray and Kirschner, 1989; Glotzer et al., 1991). However, more recent experiments suggest that the degradation of an alternative target(s) is required for the initiation of anaphase while the degradation of cyclins is necessary for the exit from mitosis after the completion of anaphase (Holloway et al., 1993; Surana et al., 1993). This degradation of cyclins and other unidentified targets is apparently ubiquitin-dependent (Glotzer et al., 1991; Holloway et al., 1993).

The budding yeast *Saccharomyces cerevisiae* has been an excellent model system to identify and characterize components that mediate the timing of anaphase in the cell cycle. Many yeast genes encoding cyclins have been identified by genetic screens or by exploiting the conservation of cyclins (Ghiara et al., 1991; Fitch et al., 1992; Richardson et al., 1992). Other components have been identified by taking advantage of the temperature-sensitive cell

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division cycle (cdc)[1] mutants, which at the nonpermissive temperature arrest at a specific stage of the cell cycle (Pringle and Hartwell, 1981). The *cdc16, cdc23, cdc20,* and *cdc27* mutants arrest in G2-M. Homologues of Cdc16, Cdc23, and Cdc27 proteins have been identified in several organisms and their inactivation also leads to arrest before anaphase (Culotti and Hartwell, 1971; Byers and Goetsch, 1975; Hirano et al., 1988, 1990; Sikorski et al., 1990). Insight into the molecular function of these proteins has come from several recent observations. First, some *cdc16* and *cdc23* alleles have been shown to be defective in the degradation of mitotic cyclins (Irniger et al., 1995). Second, in yeast the Cdc16, Cdc23, and Cdc27 proteins form a complex, and in *Xenopus* Cdc16p and Cdc27p homologues have been identified as components of a 20S complex with mitotic-specific ubiquitin-conjugating activity (Lamb et al., 1994; King et al., 1995; Tugendreich et al., 1995). Together these results suggest that Cdc16p, Cdc23p, and Cdc27p are part of an anaphase promoting complex (APC) which ligates ubiquitin onto mitotic cyclins and other unidentified proteins, targeting them for degradation (Irniger et al., 1995; King et al., 1995; Tugendreich et al., 1995). The Cdc20 protein of budding yeast may also be involved in the degradation of cyclins or other mitotic products by inference from studies of a Cdc20-like protein from *Drosophila* (Dawson et al., 1995).

The onset of anaphase is also controlled by regulatory systems called checkpoints that arrest cell cycle progression in response to various types of intracellular damage (reviewed in Hartwell and Kastan, 1994). Both DNA and spindle damage can activate checkpoints that arrest cell division in G2-M by inhibiting the onset of anaphase, cytokinesis, and new rounds of DNA replication. In budding yeast components of these checkpoints have been identified through the isolation of mutants that fail to arrest the cell cycle in response to damage. The DNA damage checkpoint(s) requires products encoded by *RAD9, RAD17, RAD24, MEC1, MEC3,* and *RAD53 (MEC2/SAD1)* genes while the spindle-damage checkpoint(s) requires a different set of products encoded by *BUB1, BUB2, BUB3, MAD1, MAD2,* and *MAD3* genes (Weinert and Hartwell, 1988, 1993; Hoyt et al., 1991; Li and Murray, 1991; Allen et al., 1994; Weinert et al., 1994). The existence of two distinct set of proteins is not surprising given that these checkpoints must respond to different types of damage. However, it is likely that these different components of the spindle- and DNA-damage checkpoints ultimately interact with common components which in turn mediate inhibition of anaphase, cytokinesis or new rounds of DNA replication. Support for this idea comes from the the recent demonstration that p53 participates in both the DNA- and spindle-damage checkpoints in mammalian cells (Cross et al., 1995).

We began a study of the onset of anaphase in budding yeast by studying one specific aspect of this process, the dissolution of pairing between sister chromatids. The inability to resolve individual chromosomes of budding yeast by standard cytological methods precludes the use of these methods to follow sister chromatid association. However, the proximity of specific chromosomal sequences on sister chromatids can be monitored by fluorescent in situ hybridization (FISH) (Guacci et al., 1994). Using FISH we have shown that sister chromatids are tightly associated along their arms and at the centromere as they are in other organisms and that pairing seems to be established concomitant with DNA replication (Guacci et al., 1994). We have also isolated mutants that lose viability when transiently arrested in mitosis by the microtubule inhibitor benomyl but not when transiently arrested in G1 with the mating pheromone α factor (Guacci et al., 1993). These mitotic conditional lethal (mcl) mutants presumably identify defects in structural or regulatory functions of mitosis including sister chromatid cohesion. Here, we describe the analysis of one mutant from this collection, *pds1* (initially referred to as *dsc1*; Guacci et al., 1993) that exhibits precocious dissociation of sister chromatids. We show that Pds1p is an important regulator of the onset of anaphase both for the APC and checkpoint pathways.

Materials and Methods

Yeast Strains and Media

Yeast strains and genotypes are shown in Table I. YPD media contained 1% yeast extract, 2% peptone, and 2% glucose. Yeast transformations and genetic manipulations were as described previously (Rose et al., 1990). For the construction of the deletion of the *PDS1* gene (*pds1-Δ2*) see Yamamoto, Guacci, and Koshland (accompanying manuscript).

1. *Abbreviations used in this paper*: APC, anaphase promoting complex; cdc, cell division cycle; FISH, fluorescent in situ hybridization; mcl, mitotic conditional lethal.

Table I. Strain List

Strain	Genotype	Source
801-6-1	*MATa ade3 trp1 bar1 fall*	Guacci et al., 1993
810-15-1	*MATα ura3 his3 Δ200 CUP1*	Guacci et al., 1994
701A-3-4	*MATa pds1-1 ade3 trp1 bar1 gal1*	This study
820-4C	*MATα pds1-1 ura3 CUP1*	This study
661-1-4	*MATa pds1-1 ura1 gal1*	This study
4530-161	*MATα cdc16-1 leu2 ade2 ade3 his7 can1 sap3 gal1*	
4531-4-2	*MATα cdc20-1 leu2 ade2 ade3 his7 ura1 can1 sap3 gal1*	
CP16-2	*MATa pds1-Δ2 cdc16-1 his7 hom3 can1 sap3 gal1*	This study
CP20-5	*MATa pds1-Δ2 cdc20-1 his7 hom3 can1 sap3 gal1*	This study
874-6C	*Matα cdc13-1 pds1-2 leu2 can1 ura3 ade2 ade3 his3 his7*	This study
874-4B	*Mata cdc13-1 pds1-2 leu2 can1 his3? his7?*	This study
H13cla5	*Mata cdc13-1 his7 can1 hom3 sap3 gal1*	
5235-8-4	*Matα cdc13-1 leu2 ura3 ade2 ade3 his7 gal1*	

Reagents

Benomyl was a gift from Dupont (Wilmington, DE). 4',6'-Diamidino-2-phenylindole (DAPI), hydroxyurea (HU), and α factor were purchased from Sigma Chemical Co. (St. Louis, MO). Nocodazole (NZ) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Oxalyticase was purchased from Enzogentics (Corvallis, OR). Histone H1 was purchased from Boehringer Mannheim (Indianapolis, IN).

Viability of Wild-type and pds1 Cells Treated with Nocodazole, α Factor or Both

Cultures of wild-type (801-6-1) and *pds1-1* (701A-3-4) cells were grown to a density of ~3 × 10^6 cells/ml and subjected to different treatments with either nocodazole, α factor or both. For one treatment, nocodazole was added to these cultures (15 μg/ml final). At various times after the addition of nocodazole, an aliquot of cells was removed, and cell number was determined using a hemocytometer. At the same time serial dilutions of the cultures were plated onto YPD plates to determine the number of colony forming units in each culture. Alternatively, cultures of these cells were treated with: (*a*) α factor (10^{-6} M final) for 5 h; (*b*) α factor for 2 h followed by 3 h in both nocodazole and α factor; or (*c*) α factor for 2 h, followed by 1 h in both nocodazole and α factor, and then 2 h in nocodazole alone. At the end of these regimens cell number and colony forming units were determined for each of these cultures as described above.

Fluorescent in Situ Hybridization

Cultures of wild-type (801-6-1) and *pds1-1* (701A-3-4) cells were grown at 23°C to a density of ~3 × 10^6 cells/ml. Cultures were treated with nocodazole (15 μg/ml) for 5 h. Aliquots of cells were fixed before and after treatment with nocodazole and subjected to FISH as described by Guacci et al. (1994).

Analysis of DNA Content, Cell and Microtubule Morphology, and H1 Kinase Activity

To analyze DNA content of cells, aliquots of cells were fixed with 70% ethanol and stained with propidium (Hutter and Eipel, 1978). The DNA content of 25,000 cells in each sample were determined with a Coulter EPICS 752 flow cytometer. To assay cell and microtubule morphology, aliquots of cells were fixed with 3.7% formaldehyde. Cell morphology was scored by phase microscopy, and microtubules were visualized by indirect immunofluorescence (Kilmartin and Adams, 1984). Extracts for H1 kinase assays were prepared from exponential cultures of *pds1* (701A-3-4) or wild-type (801-6-1) cells or the same cultures grown in the presence of α factor (10^{-6} M) or nocodazole (15 μg/ml) for 4 h. The cells in these cultures were converted to spheroplasts using oxalyticase. α factor and nocodazole were present in the spheroplasting buffers of α factor and nocodazole-treated cultures, respectively. Cultures were lysed and assayed for phosphorylation of histone H1 (Hoyt et al., 1991).

Analysis of cdc pds1 Double Mutants

Single (*cdc*) and double (*cdc pds1*) mutants were grown at 19°C to a density of 3 × 10^6/ml. Hydroxyurea (0.1 M final) was added to the cultures which were incubated for 5.5 h to arrest cells in S phase. Cells were grown at 19°C because the percent of viable cells in the *pds1* cultures is higher than observed at 23°C (see Yamamoto, Guacci, and Koshland, accompanying manuscript), and S phase arrest was also better. At the end of 5.5 h, cells were shifted to 37°C for 1 h while still in the presence of HU. At the end of 1 h, an aliquot of cells was removed and fixed. The remaining cells were washed three times in YEPD (37°C) and then grown in YEPD at 37°C. Two and 3 h after removal of HU cells were fixed with formaldehyde (3.7% final concentration). Chromosome distribution, bud morphology, and spindle morphology were scored as described above.

Gamma Irradiation of pds1 Mutants

Cells were grown in YEPD at 19°C to a density of 4–5 × 10^6 cells/ml. α factor was added to a final concentration of 10^{-8} M. Cells were incubated at 19°C for 6.5 h. Cells were then spread onto YEPD plates containing 10^{-8} M and exposed to 6 Krad of γ irradiation. Cells were harvested from the plate, washed three times in YEPD plus 0.1 mg/ml Pronase, and spread onto YEPD plates. Fields of cells were photographed at 1, 4, 7, 10, and 20 h after removal of the α factor. Cells were grown at 19°C because the percent of viable cells in the *pds1* cultures is higher than observed at 23°C. Pronase was used to improve synchronous recovery from α-factor arrest.

Results

Mitotic Conditional Lethality of pds1-1

We previously described a general strategy to isolate mutants that exhibited mcl and precocious dissociation of sister chromatids (pds) (Guacci et al., 1993). By using a simple replica-patch method, a collection of temperature-sensitive lethal mutants were exposed briefly either to benomyl (a microtubule inhibitor) to generate a transient M arrest or to α factor (a mating pheromone) to generate a transient G1 arrest. Mutants were considered mcl because they exhibited qualitatively more inviability after transient exposure to benomyl compared to α factor.

We quantitated the Mcl phenotype for one mutant from this collection subsequently named *pds1-1* (see below). For these experiments we used nocodazole, a microtubule inhibitor similar to benomyl. Wild-type and *pds1-1* cells were exposed to nocodazole at 23°C for 3 or 5 h, and the percent of viable cells in the cultures was determined for each time point (Fig. 1 *A*). Two conclusions can be made from these data. First, even in the absence of nocodazole, 30–40% of *pds1-1* cells were inviable. Second, in nocodazole *pds1-1* cells lost viability much more rapidly than wild-type cells. After 5 h of nocodazole treatment, cell death in the *pds1-1* culture was 22-fold greater than in the wild-type culture. The enhanced inviability increased slightly when *pds1-1* cells were shifted to 37°C while in nocodazole (data not shown). In summary, nocodazole dramatically decreases the viability of *pds1-1* cells even at 23°C.

To address whether the death of the *pds1-1* cells in nocodazole was a specific consequence of perturbing mitosis, we examined the viability of wild-type and *pds1-1* cells when exposed to nocodazole while arrested in G1. After 3 h of exposure to α factor, nearly 100% of wild-type cells and 90% of mutant cells appeared as unbudded mononucleate cells consistent with their arrest in G1. Upon removal of α factor, the viability of wild-type or the *pds1-1* cells was unchanged compared to untreated cultures (Fig. 1, *A* and *B*; 0 min). Both wild-type and the *pds1-1* cells also remained viable when exposed to nocodazole for 3 h while they were arrested in G1 (Fig. 1 *B*). In contrast the lethality of *pds1-1* cells was five times that of wild-type cells when *pds1* and wild-type cells were exposed to nocodazole for 3 h after release from α-factor arrest (Fig. 1 *B*). These results suggest that the nocodazole-induced death of the *pds1-1* mutant requires progression through the cell cycle and is consistent with the lethal event occurring in mitosis.

To further assess the mcl phenotype of *pds1-1* cells, we followed spindle structure and chromosome segregation in wild-type and *pds1-1* cells recovering from transient exposure to nocodazole. In both wild-type and *pds1-1* cells, nocodazole treatment caused microtubule depolymerization (data not shown). When the nocodazole was removed, spindles assembled in both cell types. Once formed, the spindles elongated, and chromosomes segregated, as evidenced by two DNA masses at the poles of the elongated

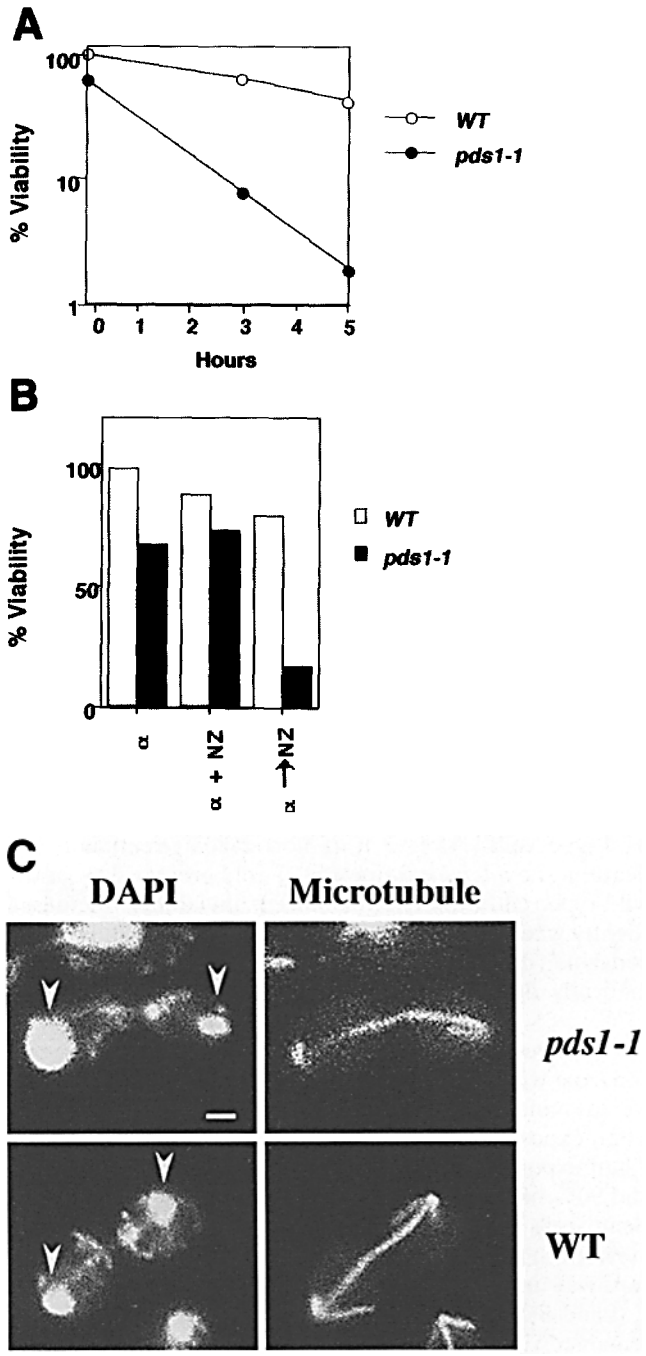

Figure 1. Effects of nocodazole on *pds1* mutants. (*A*) Nocodazole was added to exponential cultures of *pds1-1* or wild-type strains and at various times aliquots were removed to determine the % viable cells (100 × [number of cells]/[colony forming units]). (*B*) *pds1-1* and wild-type cultures were either treated with: (*a*) α factor for 5 h (α); (*b*) α factor for 2 h followed by 3 h in both nocodazole and α factor (α + NZ); or (*c*) α factor for 2 h, followed by 1 h in both nocodazole and α factor, and then 2 h in nocodazole alone (α→NZ). After these treatments the % viable cells in each of these cultures was determined as in *A*. (*C*) Wild-type or *pds1-1* cells (strain 661-1-4) were treated with nocodazole for 3 h at 23°C, then released in to fresh medium lacking nocodazole. After 1 h, cells were fixed and processed for DNA staining with DAPI and microtubule staining with an anti-tubulin antibody (Kilmartin and Adams, 1984). Arrowheads point to the segregated chromosomal mass. The smaller punctuate staining in DAPI-stained cells is mitochondrial DNA. Bar, 2 μm.

spindle (Fig. 1 *C*). In 93% of the wild-type cells the two DNA masses appeared equal in size suggesting that sister chromatids segregated properly from one another. This conclusion is in agreement with the good viability of wild-type cells after transient exposure to nocodazole. In contrast, in 42% of the *pds1-1* cells, the DNA masses at the poles of the elongated spindle differed visibly in size by more than twofold (as judged qualitatively by eye). This result suggests that gross chromosome missegregation occurred in many *pds1* cells after transient nocodazole treatment. In fact, the percentage of aneuploid *pds1* cells is likely to be greater than 42% as missegregation of only a few chromosomes would not cause a detectable difference in the visible size of the two DNA masses. The high rate of aneuploidy in *pds1-1* cells after nocodazole treatment is sufficient to explain their Mcl$^-$ phenotype.

Dissociation of Sister Chromatids in Nocodazole-treated pds1 Mutants

We began with the premise that the mitotic conditional lethal phenotype and associated chromosome missegregation might be caused by an inability to hold sister chromatids together while in nocodazole. To examine the pairing of sister chromatids in nocodazole-treated *pds1-1* and wild-type haploids, we first examined the DNA content of these cells. In both cell types ~90% cells had a 2C content indicating that they had completed DNA replication, i.e., they had sister chromatids.

Next we used FISH to ask whether the sister chromatids were associated in nocodazole-treated cells. We showed previously that a single FISH signal is observed when sister chromatids are tightly associated at that locus homologous to the probe (Guacci et al., 1994). Indeed, when nuclei from wild-type cells were probed with a centromere-proximal unique sequence from chromosome XVI, 85% showed a single FISH signal while 15% had two (Fig. 2 *A*). These results confirmed that sister chromatids are tightly associated at this locus in most wild-type nuclei. When the *pds1-1* mutant was subjected to the same regimen, nearly 50% of the nuclei exhibited two FISH signals, higher by almost 3.5-fold than that observed in wild-type cells. Similar differences were observed when wild-type and *pds1* haploids were hybridized with a probe from a centromere-proximal region on chromosome I or a centromere-distal region of chromosome XVI (data not shown). When the logarithmic culture of the *pds1-1* mutant was subjected to FISH before nocodazole treatment, only 15% of nuclei gave two signals for each of the probes. This result showed that the extra signals in the nocodazole-treated *pds1-1* cells were induced by nocodazole treatment and were not the result of pre-existing cells in the culture with disomic chromosomes. Second, the fact that 90% of the nocodazole-treated *pds1* cells had a 2C DNA content (Fig. 3 *A*) indicated that extra FISH signals were not caused by new rounds of DNA replication. We conclude that the increased number of FISH signals in the *pds1-1* mutant upon nocodazole treatment reflects a failure to initiate or maintain sister chromatid cohesion, hence we name the mutant *pds1-1* for precocious dissociation of sister chromatids.

The *PDS1* gene was cloned by complementation of the Ts lethality of the *pds1-1* mutant (see accompanying

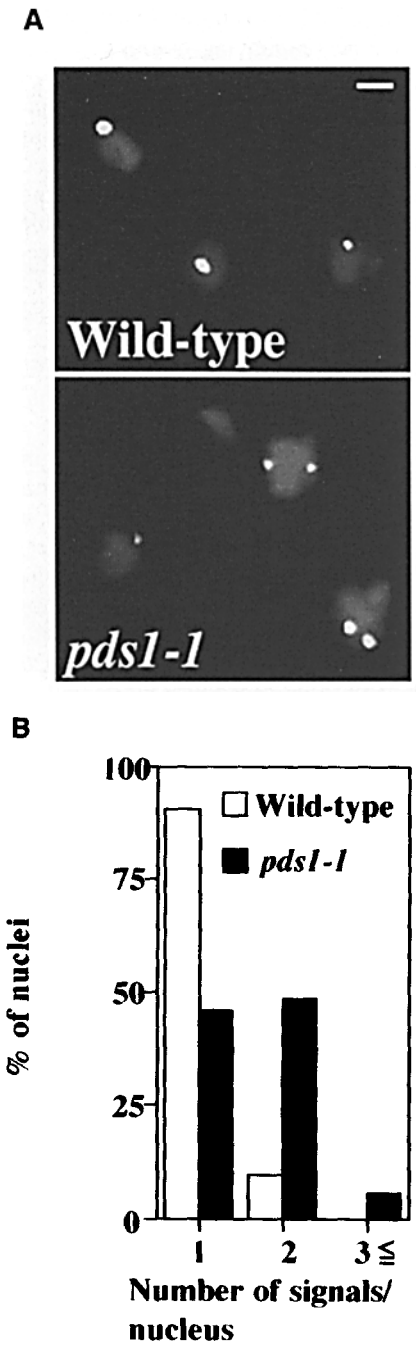

Figure 2. FISH analysis of chromosomes in wild-type and *pds1-1* haploid cells treated with nocodazole. Wild-type and *pds1* cells were exposed to nocodazole for 5 h at 23°C, fixed and then subjected to FISH (see Materials and Methods). (*A*) Micrographs of nuclei from wildtype and *pds1-1* are shown. Chromosomal DNA is stained with propidium iodide and appears gray. Centromere-proximal chromosome XVI sequences are detected by FISH and appear as white dots (Guacci et al., 1994). (*B*) The percent of nuclei with 1, 2, or 3 signals was determined from the analysis of 200 nuclei. Bar, 2 μm.

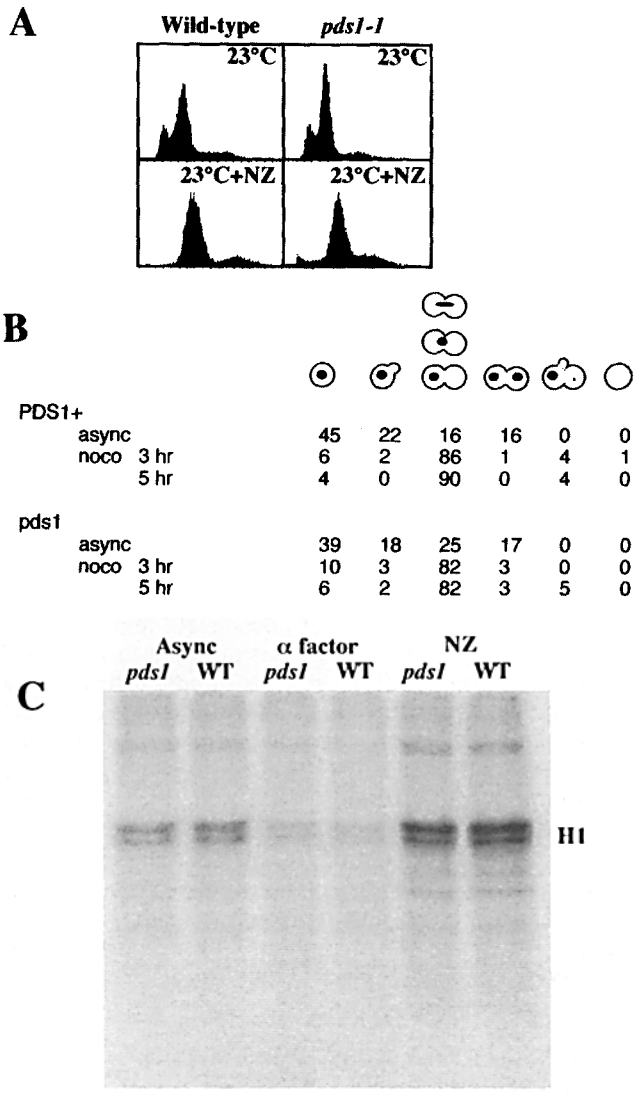

Figure 3. DNA content, cell morphology and H1 kinase in nocodazole-treated *pds1* and wild-type cells. (*A*) Flow cytometric analysis of DNA content of the cells just before (23°C) or 5 h after incubation in the presence of nocodazole (23°C+NZ). (*B*) Nocodazole-treated *pds1* cells were fixed and stained with DAPI. Cells were examined microscopically for DNA distribution and bud morphology. (*C*) Extracts were prepared from *pds1* and wild-type cells that were growing asynchronously at 23°C (Async) or treated for 5 h with either with α factor or nocodazole. Histone H1 was added to the extracts and its phosphorylation was monitored by SDS page electrophoresis (see Materials and Methods).

manuscript, Yamamoto, Guacci, and Koshland). It encodes a novel, highly charged protein. The nucleotide sequence of the *pds1-1* allele reveals a premature stop codon that terminates the *PDS1* open reading frame at approximately two-thirds its normal length (O. Cohen-Fix, unpublished results). A deletion allele, *pds1-Δ2*, was constructed that removed almost the entire open reading frame. A *pds1-Δ2* haploid was viable at 23°C and had phenotypes very similar to the original *pds1-1* allele including Ts lethality, nocodazole-induced death and precocious dissociation of sister chromatids. Therefore, Pds1p is not essential for viability at 23°C. Almost all the experiments we describe below have been done with both the *pds1-1* and *pds1-Δ2* alleles, but we have not found any significant difference in the phenotypes of these two alleles.

pds1 Mutants Do Not Exit Mitosis in the Presence of Nocodazole

One possible explanation for precocious dissociation of sister chromatids in the nocodazole-treated *pds1-1* cells is that these cells fail to arrest cell cycle progression as has been observed for *bub* and *mad* mutants. To test this possibility, other parameters of cell cycle progression were monitored in wild-type and *pds1-1* haploids after 3 h of exposure to nocodazole. Most wild-type cells arrested with a 2C DNA content, a single large bud, and high H1 kinase activity (Fig. 3) as expected from previous studies of nocodazole-treated yeast (Jacobs et al., 1988). A limited percent of cells might have exited mitosis as evidenced by a small peak of cells with greater than 2C content and a small percent of cells with extra buds. The majority of *pds1-1* cells were also large budded with a single chromosome mass. Notably absent were many cells with gross aneuploidy (cytokinesis without mitosis), a single small bud (cytokinesis followed by new bud formation) or multiple buds (new bud formation without cytokinesis). These results demonstrated that in nocodazole most *pds1* cells remained blocked for cytokinesis and bud formation. As we noted above, the DNA content of *pds1* cells was indistinguishable from wild type, indicating that most *pds1-1* cells also remain blocked for new rounds of DNA replication. Finally, the level of H1 kinase activity was similarly high in both wild-type and *pds1-1* cells (Fig. 3 *C*). Therefore, by these criteria *pds1-1* mutants do not exit mitosis or initiate a new cell cycle in the presence of nocodazole. Thus, the dissociation of sister chromatids in nocodazole-treated *pds1* mutants apparently reflects either a structural defect in sister chromatid cohesion or a failure to inhibit the onset of anaphase but not other aspects of cell division.

Pds1p Is an Inhibitor of Anaphase in cdc16, cdc20, and cdc13 Arrested Cells

We wanted to test whether Pds1p was needed to inhibit anaphase (or sister chromatid separation) only in nocodazole or whether it is important for anaphase inhibition in other G2-M arrested cells. Arrest in this window of the cell cycle occurs when *cdc16*, *cdc20*, *cdc23*, or *cdc13* are shifted to 37°C. To test the role of Pds1p in the arrest of these mutants, we began by constructing *pds1 cdc* double mutants. All *cdc* and *cdc pds1* double mutants were synchronized in S phase by growing them at the permissive temperature (19°C) in the presence of hydroxyurea (HU). These cells were shifted to 37°C to inactivate the *cdc* product. Subsequently, they were placed in fresh media at 37°C lacking HU, which allowed them to exit S phase. The chromosome distribution, the spindle morphology and bud morphology were examined in the single and double mutants before and after release from S phase arrest.

The synchrony in S phase allowed us to circumvent several problems associated with the *cdc* and *pds1* mutations. In the absence of synchrony a significant percent (~10) of telophase cells accumulate because the function of these Cdc proteins is required in late telophase as well as G2-M (Irniger et al., 1995). In addition, *pds1* mutations cause a temperature-sensitive defect in spindle elongation which results from a temperature-sensitive step in the preceding G1 around the time of spindle assembly (Yamamoto, Guacci and Koshland, see accompanying manuscript). In the absence of synchrony many *pds1 cdc* doubles fail to elongate their spindles at 37°C not because anaphase is inhibited but because they progressed through the previous G1 at 37°C and incurred a *pds1*-related spindle defect. Therefore, in asynchronous *pds1 cdc* cultures at 37°C, the presence or absence of elongated spindles is not a reliable marker for the initiation or arrest of anaphase, respectively. By synchronizing the double mutants in S at 19°C, cells have all passed through G1 at 19°C eliminating the spindle elongation defect associated with *pds1*. Hence, when these cells are released from HU and shifted to 37°C, the appearance of elongated spindles becomes a reliable marker for anaphase.

The results from these experiments are summarized in Table II and Fig. 4. When wild-type cells are treated with HU, they arrest with a large bud, an undivided nucleus, a single chromosome mass of unreplicated chromosomes, and a short spindle. For all *cdc* and *cdc pds1* mutants, ~80% or more of the cells had this expected arrest phenotype, indicating good initial synchronization. The heterogeneous size of the buds in HU-arrested *cdc23* and *cdc20* strains probably reflected the fact that these strains were sick even at the permissive temperature. Upon release from HU, all of the single *cdc* mutants were expected to complete S phase and arrest in G2-M window of the cell cycle as previously defined for the particular *cdc* mutation. Indeed these mutants showed the phenotypes characteristic for arrest in this window, uniformly large bud, a single undivided nucleus and a short spindle. Very few of the cells progress to telophase as evidenced by the absence of budded cells with segregated chromosomes at the poles of an elongated spindle. Very few cells also underwent cytokinesis as evidenced by the small percentage of unbudded cells. In contrast, in all *cdc pds1* double mutants, telophase cells were observed frequently (from 30–60%), an increase of 10–50-fold compared to the single *cdc* mutants. For comparison when a CDC^+ *pds1* mutant was subjected to the same regime, ~70–80% of the cells underwent anaphase (data not shown). These results suggest that Pds1p plays an important role in blocking anaphase in *cdc16, cdc20, cdc23,* and *cdc13* arrested cells.

The small percent (<4) of unbudded cells in the cultures of the *cdc16 pds1, cdc20 pds1,* and *cdc23 pds1* double mutants suggest that these cells do not exit mitosis or undergo cytokinesis similar to our previous observation for nocodazole-treated *pds1* cells (Table II). Consistent with this conclusion, the H1 kinase activity in *cdc16 pds1* was high as in *cdc16* strains (data not shown), characteristic of a failure to exit mitosis. In contrast, a significant percent of *cdc13 pds1* cells were unbudded, a product of exit from mitosis and cytokinesis (Table II). In addition, half of the *cdc13 pds1* cells in the divided nuclei class (i.e., 20% of the total) had acquired a new bud(s) eminating from either the mother cell, the bud or both. These cells were placed in the divided nuclei class (as opposed to an aggregate of an unbudded and small budded cell) because the large bud could not be separated from the mother cell by sonication suggesting that cytokinesis was not complete. Nonetheless, the presence of extra buds suggested that these cells had exited mitosis. This result suggests that in *cdc13* arrested

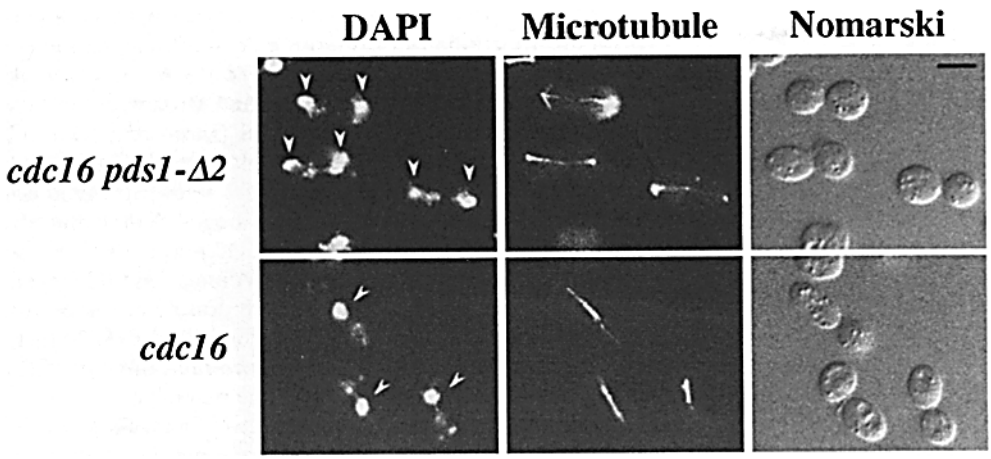

Figure 4. Micrographs of cdc16 pds1 and cdc16 cells after inactivation of Cdc16p function. H16c1a5 (*MATa cdc16-1 his7 hom3 can1 sap3 gal1*) and CP16-2 (*MATa pds1-Δ2 cdc16-1 his7 hom3 can1 sap3 gal1*) were arrested in S phase with hydroxyurea and then incubated at 37°C for 1 h to inactivate the Cdc16p function. Cells were subsequently released from S phase at 37°C by placing them into fresh medium lacking hydroxyurea. 2 h after release, the cells were fixed and processed for DNA staining with DAPI and microtubule staining with anti-tubulin antibody. Arrowheads point to the segregated chromosomal mass. The weaker punctuate staining observed in cells treated with DAPI is mitochondrial DNA. Bar, 5 μm.

cells Pds1p may play an additional important role as an inhibitor of general cell cycle progression as well as anaphase.

Pds1p Is an Inhibitor of Anaphase and General Cell Cycle Progression in γ-irradiated Cells

The arrest of *cdc13* mutants results from the activation of a DNA damage checkpoint (Weinert and Hartwell, 1993). The failure of *pds1 cdc13* mutants to arrest suggests that *pds1* mutants might be defective in this DNA-damage checkpoint. If this were the case, then *pds1* mutants should fail to arrest under other conditions that activate this checkpoint, including γ irradiation.

Wild-type and *pds1-1* cells were arrested in G1 with α

Table II. Analysis of Cell Cycle Arrest in cdc and cdc pds1 Mutants

				Large budded cells	
	Time after release from HU	Unbudded	Small budded cells	Undivided nuclei	Divided nuclei
	h				
cdc 16	0	2.4	13.4	77.2	7.1
	2	0	0	98.1	1.9
	3	0	0	91.1	3.6
cdc16 pds1-Δ2	0	8.4	11.6	71.0	9.0
	2	3.8	1.5	34.4	60.3
	3	1.4	0.7	40.0	57.2
cdc20	0	6.7	63.0	28.1	2.2
	2	4.5	27.0	68.5	0
	3	0	19.4	80.6	0
cdc20 pds1-Δ2	0	10.3	37.5	43.4	8.8
	2	2.4	4.1	33.3	57.7
	3	2.3	0.8	44.4	50.4
cdc23	0	4.7	30.7	55.9	8.7
	2	0.9	1.8	96.3	0.9
	3	3.4	0.9	92.2	3.4
cdc23 pds1-Δ2	0	5.6	24.1	62.1	8.1
	2	1.5	10.9	63.5	24.1
	3	1.7	1.7	63.6	33.1
cdc13	0	2.0	5.0	90.0	3.0
	2	3.0	0	87.0	4.0
cdc13 pds1-Δ2	0	4.0	4.0	84.0	8.0
	2	13.0	8.0	35.0	40.0

cdc and *cdc pds1-Δ2* strains (see Table I) were arrested in S phase at 23°C by treatment with hydroxyurea and then incubated at 37°C for 1 h to inactivate the *cdc* function. Cells were subsequently released from S phase by placing them into fresh medium (at 37°C) lacking hydroxyurea. Aliquots were removed just before release from hydroxyurea arrest (0 h) and 2 and 3 h after release. These aliquots were fixed and processed for DNA staining with DAPI. The cell morphology and DNA distribution was scored for 100 cells in each aliquot. ND, not done.

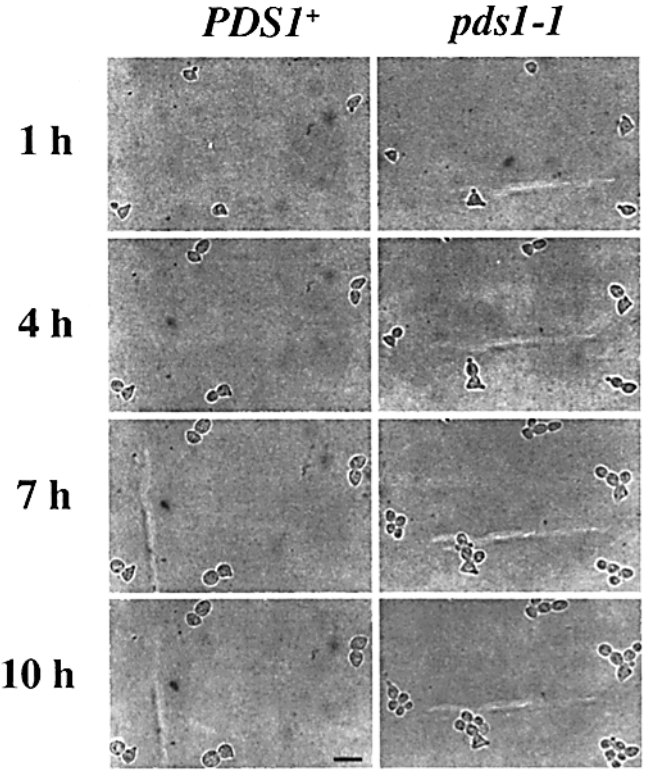

Figure 5. Photomicrographs of γ-irradiated wild-type and *pds1-1* cells. Wild-type (strain 801-6-1) and *pds1* (strain 701A-3-4) cells were arrested in G1 with α factor and then irradiated. After irradiation, cells were immediately released from G1 arrest by transferring them to solid medium. The same fields of cells were photographed at 1, 4, 7, and 10 h after irradiation. Bar, 5 μm.

factor and irradiated with γ rays to generate double strand breaks. Cells were released from the G1 block by removing the α factor and then cell division was followed by time-lapse microscopy. Previous studies showed that when wild-type cells are subjected to this regimen, they progress to G2-M and then arrest due to a DNA damage checkpoint (Chlebowicz and Jachymczyk, 1979; Brunborg et al., 1980; Weinert and Hartwell, 1988, 1990, 1993; Hartwell and Weinert, 1989; Schiestl et al., 1989; Weinert et al., 1994). Consistent with these observations our irradiated wild-type cells arrested with a uniformly large bud, an undivided nucleus and a short spindle and maintained this arrest for 20 h (Fig. 5 and Fig. 6 *A* and data not shown). In contrast, irradiated *pds1* cells formed microcolonies (Fig. 5 and Fig. 6 *A*). Cells in the microcolony had approximately normal amounts of DNA as monitored by DAPI and had array of normal spindle structures including those characteristic of telophase (data not shown). These results demonstrate that irradiated *pds1* cells undergo mitosis, that is they fail to inhibit anaphase. Furthermore, from the formation of microcolonies we infer that irradiated *pds1* cells must also undergo multiple rounds of cytokinesis, DNA replication and bud emergence. Similar results were obtained with the *pds1-Δ2* mutant (data not shown). Therefore, Pds1p is required to mediate all aspects of cell division arrest induced by γ irradiation. These results coupled with the *cdc13 pds1* results suggest that Pds1p is a component of the DNA damage checkpoint.

However, *pds1* cells do delay cell cycle progression in response to irradiation (Fig. 6 *B*). At 4 h many irradiated *pds1* cells remained large budded (two cell bodies) while almost all nonirradiated *pds1* cells had already completed cell division and had budded again (three and four cell bodies). As large budded cells are characteristic of both S phase and G2-M arrested cells, the delay observed for *pds1* cells may be in either of these stages. Other mutants defective in this DNA damage checkpoint also exhibit some residual cell cycle delay (Weinert and Hartwell, 1988). It will be informative to make double mutants with *pds1-1* and mutations in previously identified DNA checkpoint genes to assess whether their products have partially redundant functions in DNA damage response.

The inability of checkpoint-defective mutants to arrest in G2-M in response to DNA damage makes them more sensitive than wild-type cells to DNA damaging reagents (Hartwell and Weinert, 1993; Allen et al., 1994; Weinert et al., 1994). To determine whether this was also the case for *pds1* cells, we examined the viability of wild-type and *pds1* cells exposed to increasing doses of γ irradiation (Fig. 6 *C*). We observed that *pds1* cells were significantly more sensitive than wild-type cells. The radiation sensitivity was eliminated by the introduction of a plasmid with the *PDS1* gene (data not shown). We conclude that Pds1p is important for the survival of yeast cells irradiated with γ rays as expected if it is a checkpoint defective mutant.

Discussion

In this study we report the isolation of the *pds1-1* mutant of *Saccharomyces cerevisiae* by a screen for inviability after transient exposure to microtubule inhibitors and precocious dissociation of sister chromatids in the presence of these microtubule inhibitors. By following two landmarks for anaphase, sister chromatid separation and spindle elongation, we show that *pds1* mutants initiate anaphase under conditions where anaphase is inhibited in wild-type cells. These conditions include microtubule depolymerization induced by nocodazole treatment, γ irradiation, and inactivation of four different cell-division-cycle proteins (Cdc13p, Cdc16p, Cdc20p, or Cdc23p). The inappropriate initiation of anaphase in *pds1* mutants under all these different conditions suggests that the Pds1p is an inhibitor of anaphase. Furthermore, by combining our results with the current understanding of these *CDC* proteins and checkpoints, we suggest that Pds1p plays a critical role in controlling the onset of anaphase both in the APC and checkpoint pathways.

Pds1p and APC

The conclusion that Pds1p is an important anaphase inhibitor in the APC pathway is based upon genetic interaction of *pds1* mutations and temperature-sensitive *cdc16, cdc23,* and *cdc20* mutants. At the nonpermissive temperature, these *cdc* mutants arrest in G2-M before anaphase. Cdc16p is a component of APC (Irniger et al., 1995; King et al., 1995; Tugendreich et al., 1995). Several lines of evidence indicate that Cdc23p is also likely to be a component of APC. First, Cdc23p physically interacts with two established

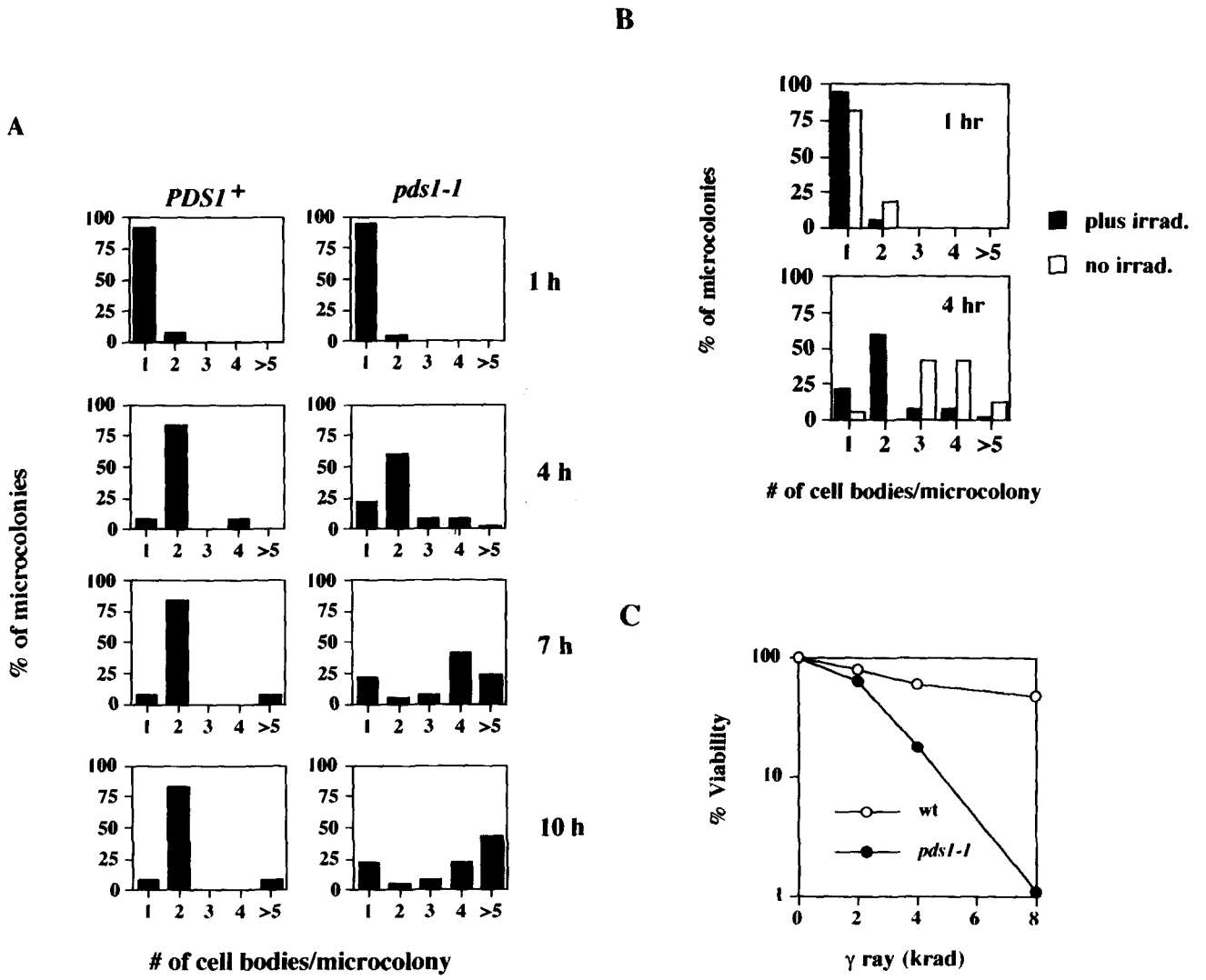

Figure 6. Cell division and viability of wild-type and *pds1* cells after γ irradiation. (*A*) Wild-type and *pds1-1* cells were plated on solid medium and exposed to γ-rays (see legend to Fig. 5). Fields of irradiated cells were photographed at 1, 4, 7, and 10 h, and the number of cell bodies in microcolonies was counted. 19 wild-type and 32 *pds1* microcolonies were scored. (*B*) *pds1* cells were irradiated as described in *A*. The number of cell bodies was determined at 1 and 4 h (*plus irrad.*) and compared to the number of cell bodies observed for mock irradiated cells (*no irrad.*) at the same time. (*C*) Asynchronous cultures of wild-type and *pds1* cells were irradiated with increasing dose of γ irradiation. Cells were plated and incubated at 23°C for 3 d. Colonies were counted and viability was determined by normalizing to the number of colonies observed in the absence of irradiation.

APC components, Cdc16p and Cdc27p (Lamb et al., 1994). In addition, Cdc23p function is required for initiation of anaphase and for the degradation of cyclins as is the function of Cdc16p (Irniger et al., 1995). While Cdc20p has not been demonstrated to associate with known APC components, it also appears to be required for initiation of anaphase and the degradation of cyclins, consistent with a function in APC (Dawson et al., 1995; Pringle and Hartwell, 1981). Unlike the single *cdc* mutants, the *cdc16 pds1, cdc23 pds1,* or *cdc20 pds1* double mutant completes anaphase as evidenced by elongated spindles and segregated chromosomes (present study). The fact that the cell-cycle-arrest phenotype of several different APC defective mutants can be altered by *pds1* mutations suggests that Pds1p is in the APC pathway.

Insight into the function of Pds1p in the APC pathway comes from recent experiments elucidating the function of APC. From these studies, APC appears to mediate ubiquitin-dependent degradation of proteins during mitosis (Holloway et al., 1993; Surana et al., 1993). The authors suggest that the APC-dependent degradation of unidentified proteins leads to the initiation of anaphase while APC-dependent degradation of cyclins allows exit from mitosis. APC-defective cells like *cdc16* mutants are thought to arrest before anaphase because they can not degrade either the factor(s) inhibiting the initiation of anaphase or the cyclins necessary for exit from mitosis. If this is the case, inactivation of the anaphase inhibitor in APC-defective cells should allow these cells to complete anaphase. However, these cells will arrest at the end of mitosis (telophase) due to their inability to degrade cyclins. Since the introduction of *pds1* mutations into APC-defective cells

(like *cdc16* mutants) causes telophase arrest (present study), *pds1* mutations cause the properties expected for inactivation of this hypothetical anaphase inhibitor. Therefore, we suggest that Pds1p is an excellent candidate for an anaphase inhibitor that is a direct or indirect target of APC.

Several findings are consistent with Pds1p being a direct target for APC degradation. Pds1p has a short amino acid sequence with similarity to cyclin degradation box, a known target for ubiquitination by APC (Glotzer et al., 1991; Yamamoto, Guacci, and Koshland, accompanying manuscript). Furthermore, using an overproducing strain to detect Pds1p, we showed that Pds1p is degraded in a cell cycle-dependent manner, and the profile of Pds1p instability is like the Clb2 cyclin, a known target of APC (Ghiara et al., 1991; Fitch et al., 1992; Yamamoto, Guacci, and Koshland, accompanying manuscript). However, the levels of the overproduced protein do not dissipate until telophase (Yamamoto, Guacci and Koshland, accompanying manuscript). If Pds1p degradation is necessary for the onset of anaphase, we would expect loss of Pds1p earlier in mitosis. As one possibility, we suggest that the functional pool of Pds1p is degraded at the onset of anaphase and its loss is obscured by the excess nonfunctional pool. The idea of localized degradation of mitotic regulators is supported by the findings of other studies (Andreassen and Margolis, 1994; Edgar et al., 1994; Tugendreich et al., 1995). Alternatively, our failure to observe the degradation of overproduced Pds1p at the onset of anaphase is a true reflection of the degradation pattern for physiological levels of Pds1p, indicating that APC indirectly regulates the anaphase-inhibitory activity of Pds1p.

Since APC functions in every cycle, and Pds1p is an apparent target (direct or indirect) of APC, it follows that Pds1p is also likely to be active in every cell cycle. In support of this conclusion, dividing cultures of *pds1-1* cells exhibit a 300-fold increase in the rate of mitotic chromosome loss and a dramatic increase in cell inviability at 23°C (Yamamoto, Guacci, and Koshland, accompanying manuscript). However, several lines of evidence argue against Pds1p being the only anaphase inhibitor active in every cell division. First, loss of all anaphase inhibitory activity in the cell might be expected to be lethal, yet *pds1-Δ2* (null allele) are viable, albeit sick. Second, a significant fraction of APC defective cells do in fact remain blocked for anaphase even after Pds1p is inactivated (present study). Finally, anaphase arrest in HU-treated cells is independent of Pds1p (present study). The idea that Pds1p might be one of several anaphase inhibitors active during the cell cycle has precedent from the previous discovery that many mitotic factors such as cyclins and kinesins have counterparts with at least partial functional redundancy (Fitch et al., 1992; Hoyt et al., 1992; Lew and Reed, 1992; Grandin and Reed, 1993).

Pds1p and Checkpoints

In this study we present evidence for Pds1p as an important inhibitor in a checkpoint since several *pds1* phenotypes are identical to the phenotypes of mutants (*rad9, rad17, rad24, mec1, mec3,* and *rad53 /mec2 /sad1*) with established defects in a DNA-damage checkpoint (Weinert and Hartwell, 1993; Allen et al., 1994). First, *pds1* mutants inappropriately initiate anaphase after γ irradiation and after inactivation of the Cdc13p. Second, γ-irradiated *pds1* mutants exhibit increased inviability compared to γ-irradiated wild-type cells. Third, irradiated *pds1* mutants not only fail to inhibit anaphase but also fail to inhibit cytokinesis, DNA replication and bud formation as has been observed for all *rad9*-related checkpoint mutants. These data strongly suggest that Pds1p is an important component of a DNA damage checkpoint that causes arrest in G2-M.

Previous experiments have documented the existence of a second DNA-damage checkpoint. This checkpoint inhibits DNA synthesis and anaphase initiation in response to various replication defects such as those induced by the DNA synthesis inhibitor, HU. However, HU-treated *pds1* mutants retain a high level of viability and remain inhibited for anaphase (this study, data not shown). Therefore, another anaphase inhibitor(s) besides Pds1p appears to act in this pathway. The existence of an alternative anaphase inhibitor is consistent with our analysis of *pds1 cdc* double mutants. However, previous results suggest that the DNA-damage checkpoints that cause arrest in S phase and G2/M share two common components, Mec1p and Rad53p, which are required to inhibit anaphase (Allen et al., 1994; Weinert et al., 1994). How can we reconcile this observation with ours? One possibility is that different types of DNA damage lead to the activation of Mec1p and Rad53p which in turn modulate the activity of different anaphase inhibitors depending upon the stage of the cell cycle.

In addition to its role in a DNA-damage checkpoint, Pds1p may be involved in a spindle-damage checkpoint. In the presence of nocodazole, *pds1* mutants share several phenotypes with other mutants defective in spindle-damage checkpoints. *pds1* mutants exhibit increased inviability when transiently exposed to nocodazole as has been reported for *bub* and *mad* mutants (this study; Hoyt et al., 1991; Li and Murray, 1991). Nocodazole-treated *pds1* mutants also fail to arrest at least the initiation of anaphase as evidenced by the precocious dissociation of sister chromatids (this study). Preliminary results suggest that nocodazole-treated *bub* and *mad* mutants also undergo precocious separation of sister chromatids as assayed by FISH (Guacci and Koshland, unpublished results). Based upon these similarities, Pds1p may be required as an anaphase inhibitor for a spindle-damage checkpoint. If this is the case, then Pds1p like p53 functions as a component common to both the spindle and DNA damage checkpoints.

However, *pds1* mutants differ significantly from *bub* mutants. Nocodazole-treated *pds1* mutants fail to exit mitosis (high H1 kinase activity) and remain blocked for cytokinesis, new bud formation and new rounds of DNA replication (this study). In contrast nocodazole-treated *bub* mutants have low H1 kinase activity and initiate DNA synthesis and bud formation (Hoyt et al., 1991). Differences between nocodazole-treated *pds1* and *mad* mutants also exist, but the significance of these differences are less clear since mad mutants have been tested at lower doses of microtubule inhibitor than those used to analyze *bub* and *pds1* mutants. Taken together, these results suggest that in the spindle-damage checkpoint, Pds1p acts only to inhibit anaphase while Bub and possibly Mad proteins are required for arresting many aspects of cell division. That is

Pds1p plays a more limited role in the spindle-damage checkpoint than the Bub and Mad proteins.

Conclusion

Results from this study support three roles for Pds1p: (*a*) an anaphase inhibitor that may be a target of APC; (*b*) an anaphase inhibitor and general cell cycle inhibitor in a DNA-damage checkpoint; and (*c*) a potential anaphase inhibitor in a spindle-damage checkpoint. These observations pose two important unanswered questions. The first question is, how does Pds1p inhibit anaphase? Currently we have no biochemical assays for Pds1p, and it has no significant similarity to other proteins in the database so its molecular function is unknown. While we can imagine that Pds1p is a classic regulator like an activator or inhibitor of a kinase, we cannot exclude the possibility that Pds1p regulates anaphase as a structural protein, for example as a component of sister chromatid cohesion.

The second question is, what is the relationship between, Pds1p, the APC, and checkpoint pathways? From our data, we can rule out that the DNA-damage checkpoint blocks cell cycle progression by inhibiting APC directly. If this were so, then irradiated *pds1* cells would behave like *pds1 cdc16* (APC defective) cells, i.e., undergo anaphase but fail to exit mitosis. In addition, we can rule out that DNA and spindle damage inhibit mitotic progression by identical mechanisms. This conclusion is based upon the observation that *pds1* mutants exit mitosis in response to DNA damage but fail to exit mitosis in response to nocodazole.

However even with these constraints, numerous models for the role of Pds1p in APC and checkpoint pathways are still viable. We propose one working model because it serves as a framework to integrate our results. We suggest that Pds1p is an inhibitor of anaphase that is one of several targets of APC. Loss of Pds1p in APC deficient cells allows anaphase to occur, but the presence of the other APC targets like cyclins prevents further cell cycle progression (i.e., exit from mitosis). The spindle-damage checkpoint blocks cell cycle progression by inhibiting directly or indirectly APC activity. Hence nocodazole-treated *pds1* cells (APC-inhibited) can initiate anaphase but cannot exit mitosis just like *pds1 cdc16* cells (APC-defective). The DNA-damage checkpoint blocks the cell cycle by modifying Pds1p. This modified form is a poor substrate for APC and a competitive inhibitor of other APC targets; it inhibits the degradation events needed for anaphase and exit from mitosis. In mutants lacking Pds1p, the modified form of Pds1p can not be made in response to DNA damage, and as a consequence all aspects of the cell cycle can proceed unaffected.

This speculative model is useful because it makes several testable predictions. For example, Pds1p should be a substrate of APC. In addition, modified forms of Pds1p should exist (potential candidates have been detected; Goetsch and Byers, personal communication; Cohen-Fix and Koshland, unpublished results), and the level of modified Pds1p should increase after irradiation. The model also shows how the complex phenotypes of *pds1* mutants can be explained by a simple molecular model. While further studies will be required to test the validity of this or other models, it is clear that Pds1p acts as an important regulator of the cell cycle in the APC and checkpoints pathways.

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