Heterologous Expression of the Human Cyclin-dependent Kinase Inhibitor p21^{Cip1} in the Fission Yeast, *Schizosaccharomyces pombe* Reveals a Role for PCNA in the *chk1*⁺ Cell Cycle Checkpoint Pathway

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Fission yeast cells expressing the human gene encoding the cyclin-dependent kinase inhibitor protein p21^{Cip1} were severely compromised for cell cycle progress. The degree of cell cycle inhibition was related to the level of p21^{Cip1} expression. Inhibited cells had a 2C DNA content and were judged by cytology and pulsed field gel electrophoresis to be in the G2 phase of the cell cycle. p21^{Cip1} accumulated in the nucleus and was associated with p34^{cdc2} and PCNA. Thus, p21^{Cip1} interacts with the same targets in fission yeast as in mammalian cells. Elimination of p34^{cdc2} binding by mutation within the cyclin-dependent kinase binding domain of p21^{Cip1} exaggerated the cell cycle delay phenotype. By contrast, elimination of PCNA binding by mutation within the PCNA-binding domain completely abolished the cell cycle inhibitory effects. Yeast cells expressing wild-type p21^{Cip1} and the mutant form that is unable to bind p34^{cdc2} showed enhanced sensitivity to UV. Cell cycle inhibition by p21^{Cip1} was largely abolished by deletion of the *chk1*⁺ gene that monitors radiation damage and was considerably enhanced in cells deleted for the *rad3*⁺ gene that monitors both DNA damage and the completion of DNA synthesis. Overexpression of PCNA also resulted in cell cycle arrest in G2 and this phenotype was also abolished by deletion of *chk1*⁺ and enhanced in cells deleted for *rad3*⁺. These results formally establish a link between PCNA and the products of the *rad3*⁺ and *chk1*⁺ checkpoint genes.

INTRODUCTION

Inherent in the design of the eukaryotic cell division cycle are checkpoints that maintain the temporal order of events, the best characterized being those that ensure the dependence of cell cycle progression on the integrity of the genome (Hartwell and Weinert, 1989). Genetic analysis in the yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* has identified a number of genes whose function is essential for the correct cell cycle response to both DNA damage and

the completion of DNA synthesis. In fission yeast, the *rad* and *hus* genes effect cell cycle arrest in G2 in response to either lesion while *chk1*⁺ falls on a second pathway that monitors only DNA damage (Enoch *et al.*, 1992; Al-Khodairy *et al.*, 1994; see Murray, 1994 for review). In this respect, the *chk1*⁺ resembles the RAD9 checkpoint gene of budding yeast (Weinert and Hartwell, 1988).

In mammalian cells, cell cycle arrest in late G1 in response to DNA damage is mediated by the transcription factor p53 (Maltzman and Czyzyk, 1984; Kastan *et al.* 1991) whose tumor suppressor function is well established. Among a number of genes that are

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regulated by p53 is the cyclin-dependent kinase inhibitor gene known variously as Cip1 (Harper *et al.*, 1993), Sdi1 (Noda *et al.*, 1994), Cap20 (Gu *et al.*, 1993), Pic1 (Hunter, 1993), or Waf1 (El-Deiry *et al.*, 1993). p21^{Cip1} is a potent negative regulator of G1 cyclin-dependent kinase (cdk)-cyclin complexes (Gu *et al.*, 1993; Xiong *et al.*, 1993; Harper *et al.*, 1993, 1995; Zhang *et al.*, 1993; Dulic *et al.*, 1994; Li *et al.*, 1994), which may additionally contain proliferating cell nuclear antigen, PCNA. Although a number of studies have shown that the binding of p21^{Cip1} to PCNA selectively inhibits DNA replication in vitro (Xiong *et al.*, 1992; Zhang *et al.*, 1993; Flores-Rozas *et al.*, 1994; Li *et al.*, 1994; Shivji *et al.*, 1994; Waga *et al.*, 1994), others have shown that it inhibits both DNA replication and DNA repair (Pan *et al.*, 1995).

We recently used alanine-scanning mutagenesis of p21^{Cip1} to define its sites of interaction with both cdk and PCNA (Goubin and Ducommun, 1995). The cdk-binding domain was determined to be within the N-terminal region of the protein, amino acids 45–60, while the PCNA domain was toward the C-terminus, amino acids 142–164. Other groups have used different experimental strategies to identify the two binding domains (Chen *et al.*, 1995; Luo *et al.*, 1995; Warbrick *et al.*, 1995). To extend our analysis of p21^{Cip1} function to the cellular level, we have expressed a human cDNA encoding the wild-type Cip1 gene and mutants in both binding domains in the fission yeast *S. pombe* under the control of the *nmt* thiamine-inducible promoter (Maundrell, 1990; Basi *et al.*, 1993; Forsburg, 1993). We show here that p21^{Cip1} inhibits progress through the G2 phase of the fission yeast cell cycle by interaction with PCNA in a *rad3*- and *chk1*-dependent manner. Our results strongly suggest a role for PCNA in a DNA damage checkpoint pathway in fission yeast.

MATERIALS AND METHODS

Yeast Strains and Cultures

The *S. pombe* strains used in this study were SP199 (*h⁺N leu1-32 ura4D-18 ade6.210*), the wee strain *cdc2-3w* (Fantès, 1981), the *rad3* deletion strain (*h⁻ rad3::ura4+ ura4D-18 leu1-32 ade6.704*), the *chk1* deletion strain (*h⁻ chk1::ura4+ ura4D-18 leu1-32 ade6.704*) and the *cds1* deletion strain (*h⁻ cds1::ura4+ ura4D-18 leu1-32*; Murakami and Okayama, 1995). Cells were cultured in supplemented minimal medium, EMM (Nurse, 1975; Alfa *et al.*, 1993). *S. pombe* strains were transformed by electroporation with the pREP41 plasmid constructions containing p21^{Cip1}-WT and the mutants ASM7 and ASM18 (Goubin and Ducommun, 1995) as glutathione S-transferase (GST) fusions (Leroy *et al.*, 1994). Transformants were selected for their ability to grow on media lacking leucine, because pREP41 carries the LEU2 gene. The promoter was kept under repressed conditions by addition of 20 μ M-thiamine to both solid and liquid media (Maundrell, 1990, 1993; Basi *et al.*, 1993). Derepressed conditions were obtained in liquid culture by washing the cells three times in minimal medium and growing them in the absence of thiamine. Phenotypes were tested at 30°C for each strain.

Plasmids

The human p21^{Cip1} cDNA cloned in the pBS vector was kindly provided by D. Beach (Howard Hughes Medical Institute, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). The wild-type gene and the two mutants were subcloned as *NcoI*–*HindIII* cassettes in the vector pGEX-KG (Pharmacia, Piscataway, NJ) to allow the production of GST amino-terminal fusion proteins. The coding sequence of GST fused to p21^{Cip1} WT and mutants ASM7 and ASM18 was inserted in the polylinker of pREP41 (Basi *et al.*, 1993; Maundrell, 1993) producing an extended open reading frame of 1.7 kb (pREP41-GST-p21^{Cip1}Hs). The predicted molecular mass of GST-p21^{Cip1} is 47 kDa. Finally, the *S. pombe* PCNA cDNA cloned in the pREP1 vector was kindly provided by E. Warbrick and previously described (Waseem *et al.*, 1992).

Cytology

Indirect immunofluorescence microscopy and DAPI staining were carried out according to the method of Alfa *et al.* (1993) after 26–28 h of induction in the absence of thiamine.

Antibodies

The anti-tubulin antibody TAT1 (Woods *et al.*, 1989) and the anti-actin antibody N350 (Amersham, Arlington Heights, IL) were used for immunofluorescence microscopy at a dilution of 1:50 and 1:100, respectively. p21^{Cip1} immunofluorescence used a polyclonal antibody raised against the human p21^{Cip1} protein (a generous gift of David Beach) at 1:100 dilution. Immunoblots were probed using an anti-PCNA monoclonal antibody (Oncogene Science, Cat# NA03, 1:500 dilution) and an anti-PSTAIRE monoclonal antibody (Yamashita *et al.*, 1991) at 1:1000 dilution.

Glutathione-Sepharose Precipitation

Yeast cells were harvested by centrifugation then broken in the presence of an equal amount of cold 500 μ m glass beads and an equal volume of lysis buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 50 mM sodium fluoride, 5 mM EDTA, 0.1 mM sodium orthovanadate, 1 mM dithiothreitol, and 0.1% Triton X-100) containing the following protease inhibitors: 0.1 mM phenylmethylsulfonyl fluoride, 20 μ g/ml leupeptin, 20 μ g/ml aprotinin, 20 μ g/ml pepstatin A, 20 μ g/ml TLCK (tosyl L-lysine chloromethyl ketone), and 20 μ g/ml TPCK (tosyl phenylalanine chloromethyl ketone). The samples were vortexed six times for 30 s at 4°C. The soluble protein fraction was recovered by centrifugation for 15 min at 14,000 $\times$ g. Precipitations were carried out by incubating 1 mg of protein extract in 500 μ l lysis buffer with 50 μ l of pre-equilibrated glutathione-Sepharose for 1 h at 4°C on a rotator. The bead pellets were then washed three times with lysis buffer.

SDS-PAGE and Immunoblotting

Proteins bound to glutathione-Sepharose beads were dissolved in Laemmli sample buffer and subjected to SDS-PAGE (Laemmli, 1970). Electrophoresis onto immobilon membrane (Millipore, Bedford, MA) was performed as described by Harlow and Lane (1988) using a semi-dry transfer apparatus. Immunoblots were probed using specified antibodies. Immunodetections were performed using goat anti-rabbit or mouse horseradish peroxidase-conjugated antibodies (Sigma, St. Louis, MO) and the ECL enhanced chemiluminescence detection kit (Amersham) according to the manufacturer's instructions.

Flow Cytometry

Flow cytometry analysis was performed on a Facscan (Becton Dickinson, Mountain View, CA) as described by Moreno *et al.* (1991). The

data were collected and analyzed using the Lysis II software (Becton Dickinson).

Pulse Field Gel Electrophoresis

Plugs were prepared from cells grown in minimal medium in the presence or absence of thiamine for 32 h as follows: cells were washed twice in CSE (20 mM citrate-phosphate, pH 5.6, 1.2 M sorbitol, 40 mM EDTA) and then resuspended in 10 ml CSE with 15 mg Zymolyase-20T (ICN Biomedicals, Costa Mesa, CA). After digestion for 1 h at 37°C, cells were pelleted and resuspended at 6×10^8 cells/ml in TSE (10 mM Tris-HCl, pH 7.5, 0.9 M sorbitol, 45 mM EDTA). After warming to 37°C, an equal volume of 1% LGT agarose in TSE was added and 100 μ l aliquots dispensed to a plug mould. Cells were lysed by incubating the plugs in 0.25 EDTA, 50 mM Tris-HCl, pH 7.5, and 1% SDS for 90 min at 55°C. The plugs were then transferred to 1% lauryl sarcosine and 0.5 M EDTA, pH 9.5, with 0.5 mg/ml proteinase K and digestion was continued at 55°C for 48 h. Plugs were stored at 4°C and equilibrated in TE before loading. Gels were run at a voltage gradient of 1 V/cm for 3 days and stained with ethidium bromide.

UV Sensitivity

Cells containing the plasmids GST-p21^{Cip1}-WT and mutants were grown for 26 h in the presence or absence of thiamine, counted, and plated at ~500 cells per plate on supplemented minimal medium in the presence of thiamine. Plates were irradiated using a Phillips 6-watt TUV lamp (95% output at 253.7 nm) delivering 860 erg/cm²/s for different times. After incubation at 30°C for 5 to 6 days, colonies were scored and survival was expressed as a percentage of colonies formed without UV treatment.

RESULTS

p21^{Cip1} Inhibits Cell Cycle Progress in Fission Yeast

When fission yeast cells carrying the plasmid pREP41-GST-Cip1 were inoculated into minimal medium containing thiamine, growth was exponential with a doubling time of approximately 3 h (Figure 1A). Parallel cultures lacking thiamine initiated growth at approximately the same rate, but after about 20 h, growth was clearly compromised and the doubling time of the culture increased to approximately 13 h (Figure 1A). Similar inhibition of growth was observed when p21^{Cip1} was expressed in the same plasmid but lacking the GST fusion (our unpublished results). Microscopic examination of samples taken 24 h after thiamine depletion revealed a heterogeneous cell population, but which contained a high proportion of cells that were conspicuously longer (range = 10–42 μ m; mean cell length = 20.4 μ m) than in the controls (range = 7–14 μ m; mean cell length = 9.2 μ m) (Figure 1B). The increase in cell length with increasing time of p21^{Cip1} induction was also seen by flow cytometry (Figure 1C). By contrast to the smaller cells that had compact nuclei that stained brightly with DAPI, the nuclei of the long cells appeared diffuse in outline, a characteristic of late cell cycle arrest in *S. pombe* (Khar and Mitchison, 1989; Alfa *et al.*, 1993). Cells with diffuse nuclei also frequently revealed a “cap” of DAPI-positive material (Figure 1B) that is also seen in mutants

arrested in G2 (King and Hyams, 1982) and is probably due to the clustering of telomeres at the spindle pole body at this stage of the cell cycle (Funabiki *et al.*, 1993). Staining of arrested cells with anti-actin and anti-tubulin antibodies revealed that actin was present at both cell poles in these long cells (Figure 2A) and that they contained highly elongated cytoplasmic microtubule array (Figure 2B), both of which strongly imply that the cells were in the G2 phase of the cell cycle (Marks and Hyams, 1985; Hagan and Hyams, 1988). Determination of the DNA content of cells expressing p21^{Cip1} by flow cytometry confirmed that the majority of the long cells had a 2C DNA but with significant cohorts of small, 1C cells and long cells having a greater than 2C content (Figure 1C). The latter probably result from the continued replication of mitochondrial DNA in cells arrested for cell cycle progress (Sazer and Sherwood, 1990), and the former from cells that had ceased to grow due to the loss of the selectable LEU2 marker.

Immunofluorescence microscopy using an anti-p21^{Cip1} antibody showed that p21^{Cip1} accumulated in the nucleus (Figure 3A). Cells varied considerably in the intensity of p21^{Cip1} staining, the longest cells expressing the highest levels of p21^{Cip1} (Figure 3B). Cells expressing the protein at a low level might continue to grow, thus accounting for the continued but slow rise in cell number in the absence of thiamine (Figure 1A). Cells containing no detectable protein may have lost the plasmid (the 1C peak observed by flow cytometry; see above).

Cell Cycle Inhibition Is through PCNA and Not *cdc2*

In normal mammalian cells, p21^{Cip1} is part of a quaternary complex containing a cdk, its cyclin partner, and PCNA. To investigate the molecular associations of p21^{Cip1} in fission yeast, proteins precipitated from cell extracts with glutathione-Sepharose were analyzed by SDS-PAGE and Western blotting. The results of this analysis are shown in Figure 1D. p21^{Cip1} was mostly associated with PCNA and to a lesser extent to *cdc2*. To investigate the molecular associations of p21^{Cip1} further, fission yeast cells were transformed with two p21^{Cip1} mutants in which the *in vitro* binding of p21^{Cip1} to either PCNA or to cdk is abolished (Goubin and Ducommun, 1995; Figure 4A). Cells carrying the plasmid GST-p21^{Cip1}-ASM7 (mutation D52A; defective in cdk binding) exhibited growth inhibition at the same time as those expressing the wild-type protein but the cells were longer at 24 h of expression (up to 48 μ m, mean length = 24 μ m; Figures 4B and 5A), even though the level of expression of p21^{Cip1} was comparable in both cases. Absence of binding of p34^{cdc2} to p21^{Cip1} in this construct was confirmed by analysis of proteins bound to glutathi-

Figure 1. p21^{Cip1} inhibits cell cycle progress in fission yeast and associates to PCNA and cdc2 in vivo. Human GST-p21^{Cip1}-WT was expressed in fission yeast under the control of the thiamine repressible promoter *nmf1*. (A) Growth in presence (+T) or absence (-T) of

one-Sepharose by Western blotting (Figure 5A). In cells carrying the plasmid GST-p21^{Cip1}-ASM18 (D149A; defective in PCNA binding), the ability of p21^{Cip1} to induce cell cycle arrest in fission yeast was abolished (Figure 4B). Cultures continued to divide normally and contained no long cells (see "size" panel in Figure 4B and DAPI staining in Figure 5B). Biochemical investigation of p21^{Cip1} complexes by glutathione-Sepharose chromatography confirmed that the ASM18 mutant was completely defective in binding to PCNA although binding to cdc2 was somewhat enhanced. These complexes also contained a 31-kDa protein that also reacted with the PSTAIRE antibody (Figure 5B).

Interaction of p21^{Cip1} with PCNA Confers Sensitivity to UV

To determine whether p21^{Cip1} was interfering with PCNA's role in DNA repair, we checked the sensitivity to UV illumination of strains carrying all three p21^{Cip1} constructs (wild type, ASM7, and 18). Cells were grown in the presence or absence of thiamine for 28–30 h, plated in the presence of thiamine, and irradiated for different periods. As can be seen in Figure 6, cells grown in the presence of thiamine showed similar UV sensitivity. However, in the absence of thiamine, i.e., when p21^{Cip1} was expressed, cells carrying either the wild-type plasmid or ASM7 were significantly more sensitive to UV than ASM18. In all three stains, the three chromosomes appeared to be normally replicated as judged by pulsed field gel electrophoresis (Figure 7).

PCNA Is a Component of the chk1-mediated DNA Repair Pathway

To further investigate the effect of the interaction of p21^{Cip1} with PCNA, we expressed our various p21^{Cip1} constructs in strains having altered checkpoint responses to unreplicated or damaged DNA (Al-Khodairy *et al.*, 1994). The mutant *cdc2.3w* overrides the normal requirement for entry into mitosis on the com-

(Figure 1 cont.) thiamine. (B) DAPI staining of cells after 26 h induction of GST-p21^{Cip1}-WT (magnification = 1000 $\times$). (C) DNA content and size analysis after 8 h (top), 24 h (middle), and 30 h (bottom) following removal of thiamine. (D) Identification of proteins bound to glutathione-Sepharose. Cells were grown in the presence (lane 1 and 2) or absence (lane 3) of thiamine; total yeast proteins were extracted and 50 μ g of protein extract was loaded on the gel (lane 1). One milligram of protein extract was incubated and precipitated with glutathione-Sepharose (lane 2, +T; and lane 3, -T). Following SDS-PAGE, precipitates were analyzed by Western blotting using a mixture of two monoclonal antibodies, anti-PSTAIRE and anti-PCNA. The arrowheads indicate the position of PCNA (36 kDa), cdc2 (34 kDa), and a PSTAIRE-related protein p31 (31 kDa). The faint lower band is derived from cdc2.

Figure 2. Anti-actin (A) and anti-tubulin (B) staining of cells following p21^{Cip1} expression for 30 h. Note in particular the presence of actin at both cell poles, indicative of cells in the G2 phase of the cell cycle (magnification = 1000 $\times$).

pletion of DNA synthesis by bypassing the normal regulation of *cdc2*⁺ by *cdc25*⁺ (Enoch and Nurse, 1990). Overexpression of p21^{Cip1} resulted in the appearance of cells measuring up to 40 μ m and having a 2C DNA content. The mean cell length in parallel cultures maintained in the presence of thiamine was 5–10 μ m. A similar G2 arrest was observed in *swi7-H4*, a mutation in DNA polymerase α , and in a strain deleted for *cds1*⁺, a newly identified protein kinase thought to be involved in monitoring the completion of DNA synthesis via its interaction with DNA polymerase α (Murakami and Okayama, 1995).

A more severe phenotype was observed in strains deleted for the radiation checkpoint gene *rad3*⁺, one of a number of fission yeast genes that provide a feedback control that inhibits cell cycle progression in

Figure 3. Localization of p21^{Cip1} in *S. pombe*. (A) Human GST-p21^{Cip1}-WT was expressed in fission yeast for 28 h and immunofluorescence microscopy was performed using a polyclonal anti-p21^{Cip1} antibody. p21^{Cip1} is targeted to the nucleus (magnification = 800 $\times$). (B) The intensity of p21^{Cip1} staining was established by scanning cells of different length using a scan analysis program.

response to both DNA damage and the completion of DNA synthesis (Sheldrick and Carr, 1993; Al-Khodairy *et al.*, 1994). Overexpression of p21^{Cip1} in the absence of the *rad3*⁺ gene product was highly toxic and cells were unable to form colonies on agar plates lacking thiamine. DAPI analysis showed few long cells and small cells with a variety of mitotic defects (Figure 8A). Similar results were obtained with GST-p21^{Cip1}-ASM7 plasmid in the *rad3*⁺ deletion strain. By contrast, the inhibitory effect of p21^{Cip1} expression was markedly reduced in a strain in which *chk1*⁺, a gene that monitors just DNA damage (Walworth *et al.*, 1993; Al-Khodairy *et al.*, 1994), was deleted. In the case of the wild-type plasmid, a low level of elongated cells

were observed but the majority grew normally (Figure 8B). When GST-p21^{Cip1}-ASM7 was expressed in the absence of *chk1*⁺, no long cells were observed and growth was normal.

It has been reported that overexpression of PCNA in fission yeast results in G2 arrest (Waseem *et al.* 1992). We repeated this experiment in both wild-type cells and in strains deleted for *rad3*⁺ and *chk1*⁺. In both cases we observed the same effect as with p21^{Cip1} expression, that is, lethality in the case of *rad3*Δ and abolition of the phenotype in the case of *chk1*Δ. The effect of p21^{Cip1} expression and PCNA overexpression on cell growth in different genetic backgrounds is summarized in Table 1.

DISCUSSION

p21^{Cip1} Interacts with *cdc2* and PCNA

p21^{Cip1} and its relatives p27^{kip1} and p57^{kip2} are potent negative regulators of the G1 phase of the mammalian cell division cycle (Harper *et al.*, 1993, 1995; Xiong *et al.*, 1993; Polyak *et al.*, 1994; Toyoshima and Hunter, 1994; Matsuoka *et al.*, 1995). p21^{Cip1} interacts with two proteins that are essential for normal cell cycle progression, cyclin-dependent kinases and PCNA (Xiong *et al.*, 1992, 1993; Gu *et al.*, 1993; Harper *et al.*, 1993, 1995; Zhang *et al.*, 1993; Li *et al.*, 1994; Waga *et al.*, 1994). The binding sites for the two interacting proteins have been localized to the amino- and carboxyl-

Figure 4. Cell cycle inhibition is through PCNA and not *cdc2*: (i) growth analysis and flow cytometry. (A) Diagram of the p21^{Cip1} molecule indicating the putative cyclin-dependent kinase and PCNA interaction domains (based on Goubin and Ducommun, 1995). The protein sequences of mutants that abolish either cdk or PCNA-binding are indicated. The putative bipartite nuclear localization sequence is underlined. (B) Cells carrying the plasmid GST-p21^{Cip1}-ASM7 (defective in cdk binding) or GST-p21^{Cip1}-ASM18 (defective in PCNA binding) were grown in the presence (+T) or absence (-T) of thiamine and growth analyzed. (C) DNA content and size analysis of the two mutants (each pair of figures corresponds to the growth curve above) at 8, 24, and 30 h after removal of thiamine. The presence of greater than 2C DNA in the case of GST-p21^{Cip1}-ASM7 may be due to the continued replication of mitochondrial DNA (Sazer and Sherwood, 1990) or is a function of altered nuclear structure in arrested cells (Khar and Mitchison, 1989).

Figure 5. Cell cycle inhibition is through PCNA and not cdc2: (ii) cytology and biochemistry. (Left panels) DAPI staining of cells carrying the plasmid GST-p21^{Cip1}-ASM7 (A) or GST-p21^{Cip1}-ASM18 (B) after 28 h induction of the protein (magnification = 1200×). (Right panels) Identification of proteins bound to glutathione-Sepharose. Cells carrying the plasmid GST-p21^{Cip1}-ASM7 (A) or GST-p21^{Cip1}-ASM18 (B) were grown in the presence (lanes 1 and 2) or absence (lane 3) of thiamine; total yeast proteins were extracted and 50 µg of total protein extract was loaded on the gel (lane 1). One milligram of protein extract was precipitated with glutathione-Sepharose (lane 2, +T; and lane 3, -T). Following SDS-PAGE, precipitates were analyzed by Western blotting using a mixture of two monoclonal antibodies, anti-PSTAIRE and anti-PCNA. The arrowheads indicate the position of PCNA (36 kDa) and cdc2 (34 kDa).

regions of p21^{Cip1}, respectively (Chen *et al.*, 1995; Goubin and Ducommun, 1995; Luo *et al.*, 1995; Warbrick *et al.*, 1995). Fission yeast possesses a single cdk, the product of the *cdc2+* gene (Nurse and Bissett, 1981) and a single gene encoding PCNA, *pcn1* + (Waseem *et al.*, 1992). When cytosolic extracts from cells expressing wild-type GST-p21^{Cip1} were absorbed onto glutathione-Sepharose, three proteins were shown to be associated with p21^{Cip1} in vivo: PCNA, cdc2, and at a very low level, a 31-kDa species that cross-reacts with a monoclonal PSTAIRE antibody and whose origin is presently unknown. In mammalian cells, p21^{Cip1} is a markedly less effective inhibitor of cdc2 kinases than the G1 cdks, cdk2, cdk3, cdk4, and cdk6 (Harper *et al.*, 1995). The cdc2 bound to p21^{Cip1} in our experiments was not associated with p63^{cdc13}, the fission yeast mitotic cyclin B (Booher *et al.*, 1989; Moreno *et al.*, 1989) and we were unable to detect a histone H1 protein kinase associated with this fraction

Figure 6. Interaction of p21^{Cip1} with PCNA confers UV-sensitivity. Cells carrying the three plasmids were grown in minimal medium for 26 h in the presence (+T) or absence (-T) of thiamine, plated onto minimal medium containing thiamine, and exposed to increasing doses of UV. After incubation at 30°C, colonies were scored and survival was expressed as a percentage of colonies formed without UV treatment.

(our unpublished results). We are currently investigating the possibility that we are selecting a G1 cyclin-associated form of the cdc2 kinase.

The specificity of the interactions between p21^{Cip1} and its target proteins was confirmed by expressing p21^{Cip1} mutants that abolish both interactions (Goubin and Ducommun, 1995). No binding to p34^{cdc2} was observed with the mutant ASM7 (D52A), although PCNA-binding to this construct was, if anything, enhanced. By contrast, ASM18 (D149A) had no effect on cdc2 binding but completely abolished the interaction of p21^{Cip1} with PCNA. Thus, our in vivo findings confirm the in vitro characterization of these mutants (Goubin and Ducommun, 1995). More importantly, they show that p21^{Cip1} interacts with the same targets in fission yeast as it does in mammalian cells, namely, one or more cyclin-dependent kinases and PCNA. Strains expressing constructs in which binding to

Figure 7. Separation of chromosomes from cells expressing GST-p21^{Cip1}-WT and mutants by pulse field gel electrophoresis. Chromosome preparations were from the following cells: WT cells treated with 10 mM HU for 3 h (lane 1), untreated wild-type cells (lane 2), GST-p21^{Cip1}-ASM18 grown in the presence of thiamine (lane 3), GST-p21^{Cip1}-ASM18 grown in absence of thiamine for 30 h (lane 4), GST-p21^{Cip1}-ASM7 grown in the absence of thiamine for 30 h (lane 5), and GST-p21^{Cip1}-WT grown in the absence of thiamine for 30 h (lane 6).

PCNA was retained (wild type and ASM7) showed enhanced sensitivity to UV, suggesting that p21^{Cip1} might interfere with PCNA's role in DNA repair.

Inhibition of the Fission Yeast Cell Cycle by p21^{Cip1} Is through Interaction with PCNA

An increase in cell length is characteristic of cell cycle inhibition in fission yeast (Mitchison, 1957). Cells expressing p21^{Cip1} were heterogeneous with regard to length but were conspicuously longer than control cells carrying the same plasmid in repressed conditions. By immunofluorescence microscopy with an anti-p21^{Cip1} antibody, the observed heterogeneity was seen to be a reflection of the variation of p21^{Cip1} expression between individual cells. The longest cells in the culture (i.e., those most inhibited for cell cycle progress) expressed the highest levels of the protein. In cells expressing ASM7 (PCNA binding only), growth inhibition with an elongated phenotype was again observed. By contrast, cells expressing ASM18 (cdc2 binding only) showed no length increase and a normal rise in cell number. These results confirm that cell cycle inhibition by p21^{Cip1} in fission yeast is by interaction with PCNA and not cdc2.

Inhibition of the Fission Yeast Cell Cycle by p21^{Cip1} in G2

Our expectation in expressing p21^{Cip1} in fission yeast was that, if it arrested the cell cycle at all, it would be in G1, by inhibition of a G1 cdk, or in S phase, by interaction with PCNA. Flow cytometry of arrested cells, however, indicated that the great majority of cells were in fact in G2. There are a number of reliable

Figure 8. Expression of p21^{Cip1} for 26 h in *rad3Δ* (A) and *chk1Δ* (B) cells. Cells stained with DAPI are shown in the left panel (control cells grown in the presence of thiamine are shown as an insert in the lower left corner), DNA content in the right panel. Cells deleted for *rad3*⁺ show abnormally condensed and aberrantly segregated chromosomes and the appearance of a peak of cells having a 1C DNA content. The *chk1*⁺ deleted cells show normal nuclei and only rare long cells.

cytological predictors of this stage of the cell cycle in *S. pombe* including cell length, the appearance of the nucleus, and the presence of actin at both cell poles (Marks and Hyams, 1985; Khar and Mitchison, 1989; Funabiki *et al.*, 1993). All of these applied to cells expressing p21^{Cip1}. Further, pulse field gel electrophoresis of cells expressing all three p21^{Cip1} constructs (wild type, ASM7, and ASM18) revealed apparently normally replicated chromosomes in all cases. Therefore, not only did we fail to find any evidence of either a G1 or S phase effect of p21^{Cip1} expression, several lines of evidence, taken together, pointed to an effect in the G2 phase of the cell cycle.

In their analysis of the role of PCNA in the fission yeast cell cycle, Waseem *et al.* (1992) concluded that deletion of the *pcn1*⁺ gene resulted in cell cycle arrest in S phase, although, as in our experiments, the majority of cells accumulated with a 2C DNA content and an elongated phenotype. However, the two experiments are not directly comparable because Waseem *et al.* (1992) looked at germinating spores (*pcn1* null cells

Table 1. Effect of p21^{Cip1} expression on cell growth in different genetic backgrounds

Yeast strain	Construct	Growth	Phenotype
Wild type	—	++++	wild type
Wild type	wild type	+	G2 arrest
Wild type	ASM7	+	G2 arrest
Wild type	ASM18	++++	wild type
<i>cdc2.3w</i>	wild type	+	G2 arrest
<i>cdc2.3w</i>	ASM7	+	G2 arrest
<i>rad3Δ</i>	wild type	—	Mitosis advanced
<i>rad3Δ</i>	ASM7	—	Mitosis advanced
<i>rad3Δ</i>	ASM18	++++	wild type
<i>chk1Δ</i>	wild type	+++	wild type
<i>chk1Δ</i>	ASM7	++++	wild type
<i>cds1Δ</i>	wild type	+	G2 arrest
<i>cds1Δ</i>	ASM7	+	G2 arrest
Wild type	PCNA OP	+	G2 arrest
<i>rad3Δ</i>	PCNA OP	—	Mitosis advanced
<i>chk1Δ</i>	PCNA OP	++++	wild type

Growth in liquid medium of strains expressing GST-p21^{Cip1}-WT, GST-p21^{Cip1}-ASM7, or GST-p21^{Cip1}-ASM18 is expressed as follows: (++++)= normal growth; (+++)= slight inhibition of growth; (+)= strong inhibition of growth; (—)= no growth.

were inviable), which were presumably completely devoid of PCNA, whereas we looked at growing vegetative cells in which PCNA was not depleted but rather its associations were modified by binding to p21^{Cip1}.

Expression of p21^{Cip1} Activates a Checkpoint Pathway

In the course of investigating the effect of p21^{Cip1} expression in a variety of genetic backgrounds, we noticed that the p21^{Cip1} phenotype was dramatically modified if either of two checkpoint genes were deleted. *rad3*⁺ is among a number of so-called checkpoint genes that monitor both the completion of DNA replication and the repair of DNA damage (Jimenez *et al.*, 1992), whereas *chk1*⁺ (also known as *rad27*⁺) has a checkpoint function only in response to DNA damage. Although little is known about the biochemistry of these checkpoint pathways, the genetic data are consistent with a mechanism in which incompletely replicated or damaged DNA generates a signal that, in a *rad3*⁺-dependent manner, maintains the tyrosine-15 phosphorylation of *cdc2* and thus blocks cells from entering mitosis until the lesion is repaired (Enoch *et al.*, 1992; Al-Khodairy *et al.*, 1994; see Murray, 1994 for review). The *chk1*⁺ pathway is also assumed to work through *cdc2* but in a tyrosine 15-independent manner (Sheldrick and Carr, 1993; Walworth *et al.*, 1993).

The effect of p21^{Cip1} on fission yeast cells was partially suppressed by deletion of *chk1*⁺, completely so in the case of ASM7. This strongly indicates that cell

cycle arrest resulting from the association of p21^{Cip1} with PCNA works through the Chk1 protein kinase. By contrast, overexpression of p21^{Cip1} was synthetically lethal with the deletion of *rad3*⁺. Strikingly, the same interactions were observed when PCNA was overexpressed in *rad3Δ* and *chk1Δ* strains. In wild-type cells, elevated levels of PCNA caused cells to arrest in G2, confirming the earlier findings of Waseem *et al.* (1992). Deletion of *chk1*⁺ abolished this inhibition while, in the absence of *rad3*⁺, PCNA overexpression was toxic.

Mutants in other genes that have been implicated in cell cycle checkpoint pathways in fission yeast produced quite different responses. The mutation *cdc2.3w* overrides the normal requirement for entry into mitosis on the completion of DNA synthesis by bypassing the normal regulation of *cdc2*⁺ by *cdc25*⁺ (Enoch and Nurse, 1990). Overexpression of p21^{Cip1} resulted in a strikingly elongated phenotype, with cells measuring up to 40 μm being observed (compared with control cells that measured between 5 and 10 μm). These cells contained an interphase nucleus and were shown by flow cytometry to have a 2C DNA content. These results show that the G2 delay produced by p21^{Cip1} overproduction is not mediated by the checkpoint that is defective in *cdc2-3w*, strongly suggesting that p21^{Cip1} expression is interfering only with the DNA damage checkpoint pathway. Interestingly, ectopic expression of p21^{Cip1} in *swi7-H4*, a mutant in DNA polymerase α, also resulted in cell cycle arrest as it did in a strain deleted for *cds1*⁺, a gene isolated as a suppressor of *swi7-H4* and thought to be involved in the checkpoint that monitors DNA synthesis (Murakami and Okayama, 1995). Thus, even though *swi7-H4* is synthetically lethal with *rad3-136*, *swi7*⁺ and *cds1*⁺ do not lie on the pathway that results in cell cycle arrest when p21^{Cip1} binds to PCNA.

A Checkpoint Pathway that Monitors PCNA: A Model

Mammalian cells contain two distinct populations of PCNA, one that is diffusely distributed in the nucleus and readily extracted with dilute detergent and one that is tightly associated with replicons via DNA polymerase δ and is extracted only by treatment with high salt (Bravo and Macdonald-Bravo, 1987). The relationship between these two populations is cell cycle dependent (Morris and Mathews, 1989). Our results are consistent with the following model (Figure 9): the abrupt disappearance of PCNA from the nucleus at the end of S phase is an essential event for cells to proceed normally into G2. DNA damage induces the formation of protein "X" (conceivably, a yeast homologue of p21^{Cip1}) that binds to PCNA and alters the normal equilibrium between the two PCNA populations. The cell now no longer receives the

"pass into G2" signal and, instead, activates a checkpoint that arrests the cell cycle. p21^{Cip1}-dependent PCNA depletion and/or overexpression of PCNA also disrupt this equilibrium and activate the checkpoint pathway that passes via *rad3*⁺ and *chk1*⁺. The fact that *rad3* Δ cells die when p21^{Cip1} is expressed suggests that the association of p21^{Cip1} with PCNA actually results in DNA damage. *chk1* Δ cells are much less sensitive to UV damage (Al-Khodairy *et al.*, 1994) and hence tolerate p21^{Cip1} overexpression.

We envisage that *rad3*⁺ has two roles in the checkpoint pathway that is activated by the association of p21^{Cip1} with PCNA: the first is to facilitate DNA repair, the second is to activate cell cycle arrest via *chk1*⁺. Cells deleted for *rad3*⁺ can neither repair damage nor arrest cell cycle progress and die. By contrast, cells lacking *chk1*⁺ can repair damage (because *rad3*⁺ is intact) but cannot arrest, therefore the inhibitory effect of p34^{cdc2} is abolished. This work establishes for the first time a link between PCNA and the activation of a DNA damage checkpoint pathway in *S. pombe*. Moreover, it raises the possibility that expression of a fission yeast homologue of p21^{Cip1} is part of the normal response to DNA damage as it is in mammalian cells. The interactions that we have identified in this paper provide obvious ways to approach the identification of such a species. The recent finding that *rad3*⁺ is a member of the ATM-related family of proteins (Zakian, 1995) gives added interest to such a study. The fact that p21^{Cip1} interacts with the same targets in yeast as it does in mammalian cells suggests that expression of p21^{Cip1}

in fission yeast might contribute usefully to the study of this important class of cell cycle regulatory protein, especially because each of its binding functions can be examined independently of the other and because we can identify genetic interaction that modifies these associations. Finally, this system might be used to gain further insights into the role of PCNA in the fission yeast cell cycle.

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Figure 9. Model of the role of PCNA in a checkpoint pathway that monitors DNA damage. (A) In wild-type cells, in the absence of DNA damage, PCNA is released from the replication complex (Rep), at the completion of S-phase; this allows cells to enter G2 and proceed to mitosis. The *rad3*⁺ and *chk1*⁺ genes have no function. (B) DNA damage induces the formation of protein "X" (possibly a yeast homologue of p21^{Cip1}). The PCNA-X complex activates the *rad3*/*chk1* pathway leading to G2 arrest. Overexpression of either p21^{Cip1} or PCNA has the same effect and activates the DNA damage checkpoint pathway.

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