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The *SFP1* Gene Product of *Saccharomyces cerevisiae* Regulates G2/M Transitions During the Mitotic Cell Cycle and DNA-Damage Response

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ABSTRACT

In eukaryotic cells, checkpoint pathways arrest cell-cycle progression if a particular event has failed to complete appropriately or if an important intracellular structure is defective or damaged. *Saccharomyces cerevisiae* strains that lack the *SFP1* gene fail to arrest at the G2 DNA-damage checkpoint in response to genomic injury, but maintain their ability to arrest at the replication and spindle-assembly checkpoints. *sfp1*Δ mutants are characterized by a premature entrance into mitosis during a normal (undamaged) cell cycle, while strains that overexpress Sfp1p exhibit delays in G2. Sfp1p therefore acts as a repressor of the G2/M transition, both in the normal cell cycle and in the G2 checkpoint pathway. Sfp1 is a nuclear protein with two Cys_2His_2 zinc-finger domains commonly found in transcription factors. We propose that Sfp1p regulates the expression of gene products involved in the G2/M transition during the mitotic cell cycle and the DNA-damage response. In support of this model, overexpression of Sfp1p induces the expression of the *PDS1* gene, which is known to encode a protein that regulates the G2 checkpoint.

EUKARYOTIC cells execute a series of discrete events as they proceed through the mitotic cell cycle. In the yeast *Saccharomyces cerevisiae*, these events include progression through START, replication of chromosomal DNA, duplication and separation of spindle pole bodies, production of buds, segregation of chromosomes, and separation of daughter cells (Pringle and Hartwell 1981). To coordinate these events, yeast has evolved feedback mechanisms that arrest further cell-cycle progression if a particular event has failed to complete appropriately or if an important intracellular structure is defective; the arrest is maintained until the problem has been resolved, at which point the cell reenters the mitotic cycle (reviewed in Murray 1994, 1995; Elledge 1996). Examples of such feedback mechanisms include cell-cycle blocks occurring in response to inappropriate configurations, i.e., damage, to either genomic DNA or the mitotic spindle.

The intracellular systems responsive to DNA damage and spindle disruption block progression of the cell cycle at a small number of defined positions known as checkpoints (Hartwell and Weinert 1989). The feedback pathways that monitor genomic integrity, for instance, lead to cell-cycle arrests at three different checkpoints: one in late G1 at START (Siede *et al.* 1993); one in S (Paulovich and Hartwell 1995); and one in late G2 (Weinert and Hartwell 1988). The pathway that monitors microtubule structure, on the other hand, leads to a single arrest during the metaphase-anaphase transition in mitosis (Hoyt *et al.* 1991; Li and Murray 1991). In this article, we describe the identification and characterization of a new yeast gene, *SFP1*, whose product is in the pathway that blocks progression at the G2 checkpoint in response to DNA damage.

The G2 damage checkpoint has been analyzed in detail in yeast. Currently, eight genes that, when mutated, eliminate some aspect of its control have been identified. These genes can be subdivided into three classes (Elledge 1996). The first class encodes proteins that act as DNA-damage sensors. They are thought to be involved in the generation, processing, or recognition of single-stranded DNA [the *in vivo* generation of excess single-stranded DNA, a common intermediate in the recombinational and nucleotide-excision repair pathways, is the likely inducer of the G2 checkpoint arrest (Garvik *et al.* 1995)]. The genes comprising this class include *RAD9, RAD17, RAD24*, and *MEC3* (Weinert *et al.* 1994; Lydall and Weinert 1995). While it remains unclear at the molecular level how these proteins function in the checkpoint signal transduction cascade, *RAD17* encodes a putative nuclease (Lydall and Weinert 1995; Siede *et al.* 1996) and *RAD24* encodes a protein with homology to replication factor C (Griffiths *et al.* 1995), consistent with an interaction with damaged DNA.

The second class encodes transducer proteins that transmit the signal from the sensor proteins. This class includes *MEC1* and *RAD53* (Stern *et al.* 1991; Allen *et al.* 1994; Kato and Ogawa 1994; Weinert *et al.* 1994). Mec1p, a member of the PI kinase superfamily, shares

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homology with the products of several other genes, including *TEL1* in *S. cerevisiae*, *rad3*$^+$ in *Schizosaccharomyces pombe*, *mei-41* in *Drosophila melanogaster*, and *ATM* and *ATR* in *Homo sapiens* (reviewed in Elledge 1996). Rad53p is a protein kinase that is phosphorylated and activated in response to DNA damage (Navas *et al.* 1996; Sanchez *et al.* 1996; Sun *et al.* 1996).

The third class of genes encodes effector proteins that mediate the cellular response to DNA damage. Two such responses have been identified: transcriptional activation of damage-inducible genes and cell-cycle arrest at the G2/M border (Elledge 1996). An important effector gene for the transcriptional response is *DUN1*, which encodes a protein kinase (Zhou and Elledge 1993; Aboussekhra *et al.* 1996; Navas *et al.* 1996). After genomic injury, mutants lacking *DUN1* fail to activate the transcription of damage-inducible genes, but they nonetheless continue to arrest at the G2 checkpoint, suggesting that the cell-cycle block is independent of the *DUN1*-regulated transcriptional response. More recent evidence, however, indicates that the function of Dun1p may be more complex because it does appear to regulate cell-cycle arrest under some circumstances (Pati *et al.* 1997). Cell-cycle arrest is also mediated by another effector gene, *PDS1* (Cohen-Fix *et al.* 1996; Yamamoto *et al.* 1996). Mutants lacking this gene fail to block at the G2/M checkpoint in response to DNA damage, as well as to the metaphase-anaphase transition after destabilization of the mitotic spindle, suggesting that the two checkpoint pathways have some overlapping components. Pds1p is thought to mediate the spindle checkpoint by binding to another protein, Esp1p, and thereby inhibiting its activity (Ciosk *et al.* 1998). When the mitotic spindle has formed appropriately, the anaphase promoting complex is then thought to degrade Pds1p, the released Esp1 dissociates "cohesins" that connect sister chromatids, and anaphase then ensues (Ciosk *et al.* 1998). The role of Pds1p in the DNA-damage checkpoint is less well characterized.

The effector molecules mediating the G2 DNA-damage checkpoint in the fission yeast *S. pombe* are better understood. In this organism, the damage-induced arrest is regulated through post-translational modification of Cdc2 cyclin-dependent kinase (CDK), specifically through the phosphorylation of tyrosine-15 (Rhind *et al.* 1997). In its unphosphorylated form, but not its phosphorylated form, Cdc2 CDK promotes entrance into mitosis (Gould and Nurse 1989). Overexpression of Cdc25 phosphatase, which removes the phosphate group from tyrosine-15, eliminates the G2 damage checkpoint, strongly arguing that the checkpoint pathway is maintained by trapping Cdc2 CDK in its phosphorylated form (Rhind *et al.* 1997). Cdc25, in turn, is the target of, and is regulated by, Chk1 kinase (Furnari *et al.* 1997), another gene product in the DNA-damage checkpoint pathway (Walworth *et al.* 1993; al-Khodairy *et al.* 1994; Walworth and Bernards 1996).

Thus, the G2 checkpoint pathway in *S. pombe* acts through a system that functions in the normal cell cycle to block progression into mitosis. While the G2 arrest in *S. cerevisiae* is less understood at the molecular level, the idea that it may also work through proteins that negatively regulate progression during the cell cycle is an attractive one. As described below, the *SFP1* gene product is a candidate for such a protein. Mutants lacking *SFP1* not only are deficient in the G2 checkpoint, but also proceed into mitosis prematurely during a normal (undamaged) cell cycle. Sfp1p therefore appears to act as a negative regulator of transition into mitosis, both in the normal cell cycle and in response to DNA damage.

MATERIALS AND METHODS

Chemicals: Nocodazole, methyl-methane-sulfonate (MMS), and α-factor were purchased from Sigma Chemical (St. Louis). Purified Taq polymerase was kindly supplied by Dr. Millie Georgiadis (Waksman Institute). Oligo(dT) cellulose was purchased from Boehringer Mannheim (Indianapolis).

Plasmids: YEpSFP1 contains a 4427-bp *Bgl*II fragment that carries the entire *SFP1* gene cloned into the *Bam*HI site of the YEp24 yeast shuttle vector. pSFP-GFP contains the entire *SFP1* gene fused in-frame to the 5′ end of the gene-encoding green-fluorescent protein carried on plasmid pTS395 (kindly supplied by Dr. T. Stearns). pZH1 is YEp24, with the exception that the *Bam*HI/*Pvu*II fragment is replaced by a 685-bp *Bam*HI/*Pvu*II fragment carrying the promoter region from the *GAL1-GAL10* transcription unit. pGAL-SFP is pZH1 carrying the entire coding sequence for *SFP1* cloned immediately downstream of the *GAL10* promoter.

Yeast strains: All yeast strains in this study were isogenic with W303-1A (*MAT***a** *SFP1 ho ade2-1 trp1-1 his3-11, 15 can1-100 ura3-1 leu2-3,112*; Thomas and Rothstein 1989). To isolate DN1090 (*MAT***a** *sfp1*Δ*::TRP1 ho ade2-1 trp1-1 his3-11, 15 can1-100 ura3-1 leu2-3, 112*), an internal *Bam*HI-*Eco*RI fragment of *SFP1* was replaced with the *TRP1* gene by standard *in vitro* cloning techniques, and the resulting construct was substituted for the *SFP1* locus in W303-1A by one-step gene transplacement. DN1091 is identical to DN1090 with the exception that it also contains YEpSFP1. DN1092 is identical to W303-1A, with the exception that it also contains pSFP-GFP. DN1093 is identical to W303-1A, with the exception that it also contains pGAL-SFP.

Differential display for damage-inducible genes: Strain W303-1A was grown in YPD medium at 30° to midlogarithmic phase (OD_{600} = 0.5). An aliquot was removed as the $t = 0$ control, and the remaining culture was brought to 0.01% (v/v) MMS. Aliquots were removed from the culture at 30, 60, and 90 min, and RNA was prepared from the untreated ($t = 0$) and treated (t = 30, 60, and 90) aliquots. Differential display was then carried out on the four samples using the RNAimage kit (GeneHunter Corporation) according to the manufacturer's instructions. Each differential display reaction is a quantitative RT-PCR that amplifies 3′ ends of multiple mRNA transcripts in one tube (Peng and Pardee 1992). To do so, the reaction uses one primer that hybridizes to (*i.e.*, is "anchored" to) the poly(A) tail and a mixture of short primers that hybridize, under the appropriate conditions of temperature and salt, to multiple sequences, even those containing mismatches. After carrying out the amplification in the presence of radioactive nucleotides, the products were separated on a standard sequencing gel. Autoradiography was used to identify the bands that altered in response to MMS treatment.

The DNA in each band of interest was eluted from the gel, reamplified using the same primers, and cloned into pGEM-T vector (ProMega, Madison, WI). The inserts were subsequently sequenced and identified by comparison to the yeast genome database (http://genome-www.stanford.edu/saccharomyces/).

RNA procedures: RNA preparation and Northern analysis were carried out as described (Brown and Mackey 1997; Hoffman 1997).

Assays for DNA-damage sensitivity: Sensitivity to MMS was assayed as described (Prakash and Prakash 1977). To assay sensitivity to ultraviolet irradiation and gamma rays, strains were grown to midlogarithmic phase in liquid cultures, the cultures were sonicated and subjected to serial dilutions, and the dilutions were plated out onto solid growth medium. The resulting plates were exposed to the indicated amount of radiation and placed into a 30° incubator. Viable cell colonies were quantitated 5–7 days later.

Assays for the G2 DNA-damage checkpoint: To monitor G2 arrest after exposure to DNA damage, wild-type and mutant cultures were grown to midlogarithmic phase in rich medium, at which point MMS was added to a final concentration of 0.01%. Aliquots were removed at various times, fixed in formaldehyde, sonicated, and scored by light microscopy for the appearance of large-budded cells.

To monitor whether an artificial pause in late G2 suppresses the sensitivity of *sfp1*Δ cells to DNA damage, a logarithmic culture of *sfp1*Δ cells was pretreated for 4 hr with nocodazole to arrest cells at the G2/M border. MMS was then added to a final concentration of 0.5%. After incubation for various times, aliquots were removed, and the nocodazole and MMS were washed out. Viability was subsequently determined as described (Prakash and Prakash 1977). As controls, logarithmic wild-type and *sfp1*Δ cultures were treated identically with the exception that there was no pretreatment with nocodazole.

To monitor the temporal pause in G2 after DNA damage, cultures of wild-type and *sfp1*Δ cells were grown to midlogarithmic phase in YPD medium, at which point they were arrested for 3 hr at START with 5 μg/ml α-factor. After arrest, cells were collected by centrifugation and then released into fresh YPD medium containing 16 μg/ml nocodazole (Hoyt *et al.* 1991). After 5.5 hr, cells were centrifuged and resuspended in distilled water, and each of the two population of cells (wild type and *sfp1*Δ) were split into two aliquots. One aliquot was irradiated by ultraviolet light (30 J/m^2) as described (Allen *et al.* 1994), and the other aliquot was left untreated. Both aliquots were then released into fresh YPD medium. Cells were collected every 20 min, fixed in 6% formaldehyde, and stained with DAPI. Cells entering mitosis were identified by their nuclear phenotypes as described (Allen *et al.* 1994).

Fluorescence activated cell sorting analysis: Fluorescence activated cell sorting (FACS) analysis was carried out as described (Hutter and Eipel 1979).

RESULTS

The *SFP1* transcript increases after MMS treatment: To identify genes involved in the DNA-damage response, many studies have sought genes whose transcripts are induced after genomic injury. In *S. cerevisiae*, this approach has led to the identification of at least 28 damage-inducible genes (Friedberg *et al.* 1995). Two technological breakthroughs encouraged us to repeat this type of analysis in yeast. The first was the development of the differential display protocol (Peng and Pardee 1992), a powerful methodology that allows one to rapidly and sensitively screen transcripts from hundreds of genes simultaneously. The second was the completion of the sequence of the yeast genome (Mewes *et al.* 1997), which allows one to unambiguously identify transcripts from the differential display after minimal amounts of DNA sequencing. Using this approach (see materials and methods for details), we identified >20 new genes whose steady-state transcript levels appeared to increase after treatment with 0.01% (v/v) MMS, a DNA-alkylation agent.

One of these genes was *SFP1*, originally isolated by Blumberg and Silver (1991) on the basis of its ability to partially block nuclear protein localization when present on a high-copy-number plasmid. The *SFP1* gene encodes a 75-kD protein with two Cys_2His_2 zinc fingers that are homologous to similar domains in a large number of transcription factors, including the *MSN1* gene product from yeast (Estruch and Carlson 1993; Martinez-Pastor *et al.* 1996; Schmitt and McEntee 1996) and the Wilms' tumor protein from humans (Hastie 1994; Figure 1). Two characteristics of the primary sequence, however, distinguish Sfp1p from these transcription factors: first, the homology outside of the zinc-finger domains is relatively weak (Figure 1); and second, the zinc fingers in Sfp1p are separated by 37 amino acids, rather than the more typical 7–8 amino acids found in transcription factors of this type (Evans and Hollenberg 1988). Sfp1p therefore belongs to a small class of proteins with so-called "split zinc-finger" motifs; this class includes Suvar(3)7 and Teashirt from *D. melanogaster* (Reuter *et al.* 1990; Fasano *et al.* 1991) and a protein encoded by the TRS1 retrotransposon of Trypanosomes (Pays and Murphy 1987). Nonetheless, direct comparison between Sfp1p and these other split zinc-finger proteins showed no significant homology in their primary sequences. Sfp1p also contains a long poly(A)sp sequence immediately N-terminal of the zinc fingers, a domain commonly found in yeast transcription factors (Hope and Struhl 1986; Ma and Ptashne 1987).

To confirm the differential display results, we performed Northern analysis to examine the expression of *SFP1* after MMS treatment. As shown in Figure 2, the transcript increased in a roughly linear fashion for 90 min after treatment, reaching a maximum that was 6.2-fold above the steady-state level in undamaged cells. *SFP1* would therefore appear to be a new damage-inducible gene. This conclusion, however, must be tempered by the observation that *SFP1* exhibits cell-cycle regulation such that its transcript accumulates late in G2 during the normal mitotic cycle (http://genomics.stanford.edu/). In other words, the transcript is normally present at a higher level during the same stage of the cycle where cells arrest in response to DNA damage. Thus, the induction observed (Figure 2) may be attributable to a G2 arrest, rather than a *bona fide* increase in transcription after DNA damage. Nevertheless, we feel this

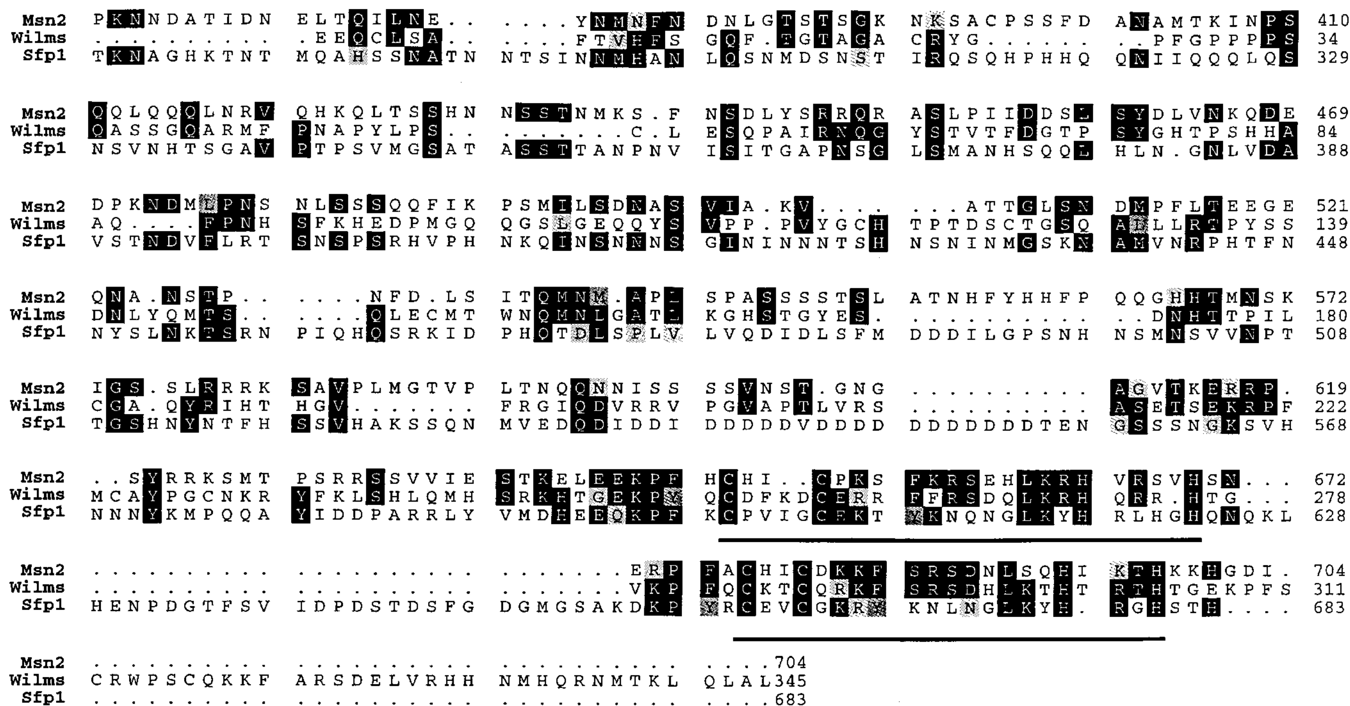

Figure 1.—The *SFP1* gene product is homologous to the *MSN2* and *WT1* gene products. Sequences were aligned and displayed using the PILEUP and PRETTYBOX programs of the Genetics Computer Group (University of Wisconsin) sequence analysis package (Devereux *et al.* 1984). Black boxes indicate identical amino acids, and gray boxes indicate similar amino acids. Shown are the entire sequence of Wilms' tumor protein and also the C-terminal portions of the Sfp1 protein and Msn2 proteins. The lines indicate the position of the respective zinc fingers, which are separated by 7 amino acids in Wilms' tumor protein and Msn2, and by 37 amino acids in Sfp1.

latter interpretation is unlikely since the steady-state levels of *SFP1* transcript fluctuate by only 2-fold in the cell cycle (http://genomics.stanford.edu/), lower than the 6-fold induction seen after DNA damage. It should also be noted that the *SFP1* transcript could be visualized only by Northern blot analysis using poly(A) transcripts—and then only weakly—indicating that *SFP1* is expressed at an extremely low level.

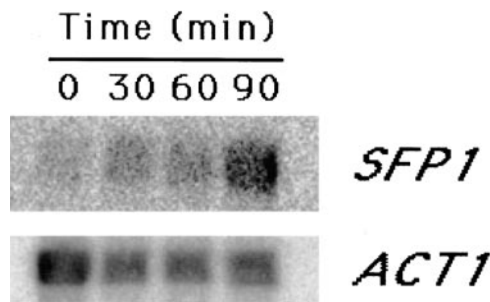

Figure 2.—Expression of *SFP1* after DNA damage. A logarithmic population of wild-type cells was brought to 0.01% MMS, and aliquots were collected at various times. RNA was extracted from the aliquots and subsequently fractionated through oligo(dT) Sepharose (Boehringer Mannheim) to select for poly(A) transcripts. The expression of *SFP1* was analyzed by Northern blotting followed by quantitation on a phosphorimager. To normalize for loading errors, the blot was stripped of old probe, rehybridized to *ACT1*, and subsequently analyzed in the same fashion.

***sfp1*Δ mutants are defective in the G2 DNA-damage checkpoint:** We next analyzed whether the *SFP1* gene product plays a role in the DNA-damage response. To do so, we compared the sensitivities of three isogenic strains to an array of DNA-damaging agents (materials and methods). Strain W303-1A, the wild-type control, contained a functional *SFP1* gene. Strain DN1090 was an *sfp1*Δ mutant, constructed by one-step gene transplacement in the W303-1A background. Strain DN1091 was isolated by transforming DN1090 with YEpSFP1, a yeast plasmid carrying a wild-type *SFP1* gene. The *sfp1*Δ mutant was more sensitive than the wild type to MMS (Figure 3A), ultraviolet light (Figure 3B), and gamma-irradiation (Figure 3C). These phenotypes were due to the *sfp1*Δ mutation since plasmid YEpSFP1 complemented the defects. The *SFP1* gene product therefore plays a role in the DNA-damage response, although its requirement appears to be relatively minor compared to other known Rad proteins (Game 1983).

As described in the next section, the *sfp1*Δ mutant has defects in the transition from G2 to M during the mitotic cell cycle. This prompted us to examine whether its sensitivity to DNA-damaging agents might result from a similar problem, specifically from an inability to arrest at the G2 checkpoint after DNA damage. To address this possibility, we carried out three different experiments. First, we directly tested whether the *sfp1*Δ mutant could arrest after DNA damage (Figure 4A). After treatment

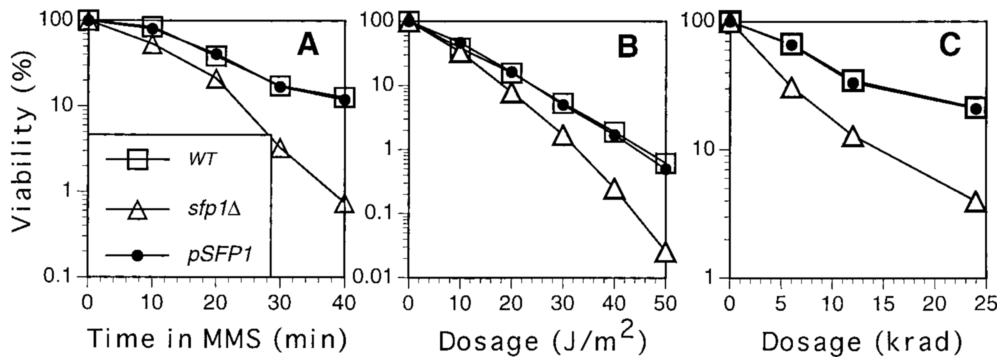

Figure 3.—The *sfp1*Δ mutant is sensitive to genomic injury. (A) The *sfp1*Δ mutant is sensitive to DNA-alkylation damage. Logarithmic cultures of wild-type cells (*WT*), *sfp1*Δ cells (*sfp1*Δ), and *sfp1*Δ cells carrying YEpSFP1 (*pSFP1*) were treated with 0.5% MMS for the indicated times. Viable cells were determined by diluting and plating onto YPD medium as described (materials and methods). (B) The *sfp1*Δ mutant is sensitive to ultraviolet irradiation. Logarithmic cultures of *WT*, *sfp1*Δ, and *pSFP1* were diluted onto YPD plates and treated with the indicated amounts of ultraviolet light. The plates were incubated at 30°, and viable colonies were quantitated 4–5 days later. (C) The *sfp1*Δ mutant is sensitive to gamma irradiation. Logarithmic cultures of *WT*, *sfp1*Δ, and *pSFP1* were diluted onto YPD plates and treated with the indicated amounts of gamma rays. The plates were incubated at 30°, and viable colonies were quantitated 4–5 days later. The *Y*-axes represent the percentage of viable cells remaining when compared to an untreated control.

of logarithmic cultures with MMS, >95% of the cells in the wild-type culture arrested at the G2 checkpoint with large buds, defined as buds with diameters at least 50% of their mother cells. By comparison, the percentage of large-budded cells did not change significantly in the *sfp1*Δ mutant after MMS treatment. The cell number of the *sfp1*Δ population increased under these conditions (data not shown), indicating that the mutant continued unimpeded through the G2 checkpoint despite the DNA damage. Moreover, the observed inability to arrest was due to the *sfp1*Δ mutation since plasmid YEpSFP1 restored normal checkpoint control (Figure 4A).

In the second experiment, we examined whether the sensitivity to DNA-damaging agents could be suppressed by blocking cells at mitosis with nocodazole, a microtuble-destabilizing agent. This type of experiment was originally designed by Weinert and Hartwell (1988) to distinguish a checkpoint mutant from a true DNA repair mutant. If a mutant's sensitivity to DNA-damaging agents is suppressed by artificially imposing a cell-cycle block, it can be concluded that the strain's repair enzymes are operable and that the original phenotype was due to a defect in checkpoint control. As shown in Figure 4B, arresting the *sfp1*Δ mutant with nocodazole at the G2/M border restored its resistance to MMS to wild-type levels. This result further supports the conclusion that the *SFP1* gene product was not required for DNA repair *per se*, but rather for the G2 checkpoint arrest.

In the final experiment, we directly determined whether the *sfp1*Δ mutant failed to pause at the G2 checkpoint after DNA damage (Figure 4C). Wild-type and *sfp1*Δ cultures were arrested in G2, irradiated, and released into fresh medium. The wild-type cells exhibited a temporal pause before entering mitosis, as expected from cells with an operational G2/M checkpoint. The *sfp1*Δ cells, however, progressed immediately into mitosis after DNA damage, demonstrating a failure of the G2 checkpoint.

The preceding three experiments showed that the *SFP1* gene product is required for the DNA-damage checkpoint in G2. The *sfp1*Δ mutant, however, arrested efficiently and synchronously in nocodazole and maintained full viability, demonstrating that its spindle checkpoint functioned normally (Figure 4C; Hoyt *et al.* 1991; Li and Murray 1991). To test whether the replication checkpoint might be compromised, we determined the viability of wild-type and *sfp1*Δ cells after treatment for 2 hr in 0.2 m hydroxyurea, a DNA chain elongation inhibitor (Elledge 1996; Navas *et al.* 1996). The *sfp1*Δ mutant was no more sensitive to this treatment than the isogenic wild-type strain was, demonstrating that its replication checkpoint remained intact.

The *sfp1*Δ mutant exhibits defects in the G2 to M transition during the mitotic cell cycle: In addition to its G2 checkpoint phenotype, the *sfp1*Δ mutant exhibited an obvious phenotype in the absence of DNA damage. Specifically, we found that it had a generation time of 190 min, compared to 90 min for the isogenic wild-type strain, in agreement with published growth rates from Blumberg and Silver (1991). This argued that Sfp1p played some role in the undamaged cell cycle. To gain more insight into this role, we examined the mutant's mitotic phenotypes in more detail.

The *sfp1*Δ mutant had one particularly unusual and intriguing phenotype during vegetative growth: it was significantly smaller than its isogenic wild-type strain (Figure 5). We photographed and measured >150 cells from logarithmically growing wild-type and *sfp1*Δ cells. The average diameter of cells in the G1 phase, defined as cells with no buds, was 28.5% smaller in the *sfp1*Δ mutant. This was particularly striking among cells that had just completed cytokinesis: the diameters of newly formed *sfp1*Δ daughter cells were up to 42% smaller than those of similar cells in a wild-type population. In addition, the average diameter of mother cells during the S + G2 + M phases, defined as cells that bore an emerging bud, was 18.7% smaller in the mutant

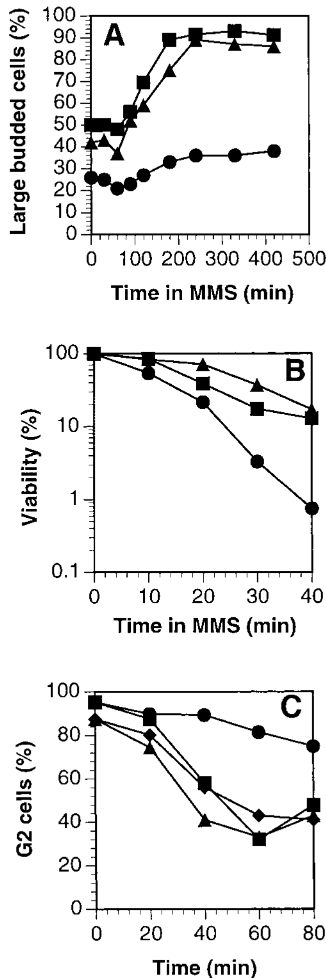

Figure 4.—The *sfp1*Δ mutant fails to arrest at the G2 checkpoint in response to DNA damage. (A) The *sfp1*Δ mutant does not arrest in G2 after DNA damage. Logarithmic cultures of wild-type cells (■), *sfp1*Δ cells (●), and *sfp1*Δ cells carrying YEpSFP1 (▲) were grown to midlogarithmic phase in rich medium, at which point MMS was added to a final concentration of 0.01%. The appearance of large-budded cells was subsequently quantitated (materials and methods). (B) Arrest in late G2 suppresses the sensitivity of *sfp1*Δ cells to MMS. Logarithmic cultures of wild-type (■) and *sfp1*Δ (●) cells were treated with 0.5% MMS and analyzed for viability (materials and methods). (▲) A second aliquot of the *sfp1*Δ strain was pretreated for 4 hr with nocodazole to arrest cells at the G2/M border and assayed for sensitivity to MMS (materials and methods). (C) The *sfp1*Δ mutant does not exhibit a temporal pause in G2 after DNA damage. Cultures of wild-type and *sfp1*Δ cells were arrested with nocodazole at the G2/M border (materials and methods). The nocodazole was washed out, and each of the two populations was split into two aliquots. One aliquot from each culture was irradiated with ultraviolet light: *WT* + *UV* (●) and *sfp1*Δ + *UV* (◆). The other aliquot was left untreated: *WT* (■) and *sfp1*Δ (▲). Both aliquots were then released into fresh YPD medium and monitored for entrance into mitosis by DAPI staining (materials and methods).

population. Therefore, bud formation and cytokinesis occurred at smaller cellular volumes in the mutant. Despite their small size, the *sfp1*Δ mutant was fully viable (data not shown). Blumberg and Silver (1991) likewise noted that *sfp1*Δ mutants released buds at a smaller size than wild-type cells did, but they also reported that a fraction of the cells in a logarithmic population had multiple buds. We observed similar multiply budded cells in our mutant, but we found that normal levels of sonication separated viable buds from the mother cell. Thus, the small daughter cells produced after cytokinesis in *sfp1*Δ mutants appear to have trouble separating from mother cells without physical disruption.

To analyze the size phenotype in more detail, we examined how cells in the mutant population were distributed through the mitotic cell cycle during logarithmic growth. Approximately 61% of cells in a mutant culture were unbudded, as compared to 25% in an isogenic wild-type control culture (budding marks the initiation of S phase). Moreover, FACS analysis indicated that the mutant and wild-type populations contained 70 and 25%, respectively, of cells with a G1 (1n) amount of DNA (Figure 5). Thus, the *sfp1*Δ mutant population was significantly skewed toward the G1 phase.

From these cell-cycle parameters, we conclude that the *sfp1*Δ mutant enters mitosis prematurely. This would account for the extremely small size at which buds pinched off from mother cells. It would also account for the apparent delay in G1 that was demonstrated by flow cytometry. Yeast cells are unable to pass through START, which is located in late G1, unless they have reached a minimum cell volume (Johnston *et al.* 1977). Therefore, the small daughter cells produced after cytokinesis in the *sfp1*Δ mutant would require extra time to reach this minimal size, thereby generating the altered cell-cycle percentages. From our measurments of mother cells, the *sfp1*Δ mutant may also pass the G1/S border prematurely, but our preceding checkpoint studies were more consistent with a specific G2/M defect. We are currently unable to account for the slightly smaller size at which G1 cells initiate budding in the mutant.

Overexpression of *SFP1* increases the percentage of budded cells: Since the underexpression of Sfp1p decreased the number of budded cells, it was of interest to determine whether overexpression of Sfp1p would have the opposite effect. To test this, we transformed wild-type cells with YEp24, a standard yeast shuttle vector, or pGAL-SFP1, a YEp24 vector carrying the *SFP1* gene under the control of a *GAL1* promoter (materials and methods). The transformants were grown under noninducing conditions (raffinose-containing media) to midlogarithmic phase, galactose was added, and aliquots were removed and scored for budding ratios (Figure 6). In the control culture, this regime had little effect on the overall number of large-budded cells. In the experimental culture, however, the percentage of cells with large buds increased from 32 to 58%. This

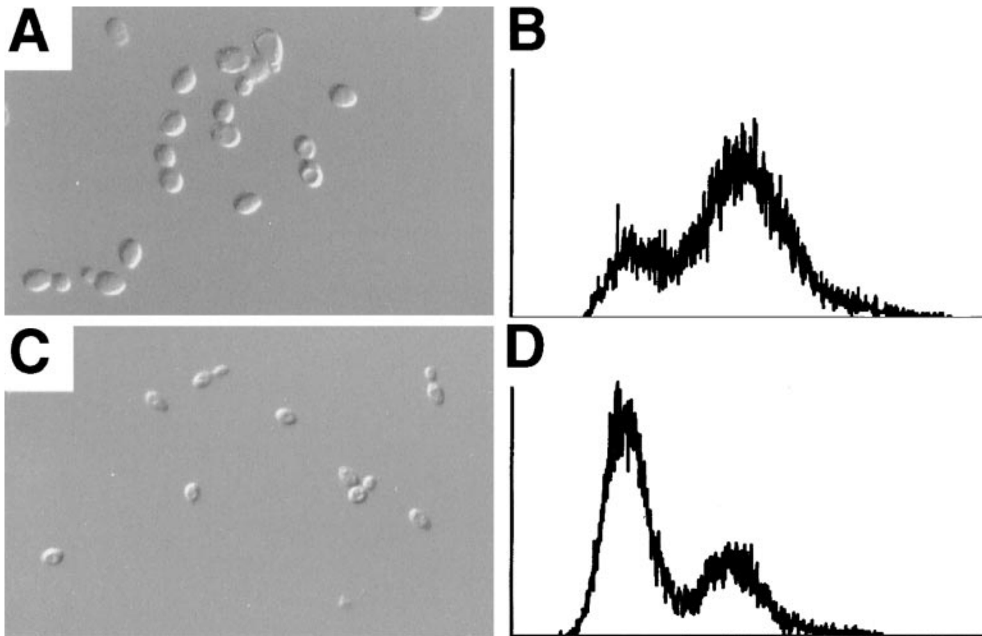

Figure 5.—The *sfp1*Δ mutant has a "Wee" phenotype. (A) Logarithmic wild-type cells viewed by Nomarski microscopy. (B) DNA content in a logarithmic population of wild-type cells determined by FACS analysis. (C) Logarithmic *sfp1*Δ cells viewed by DIC microscopy. (D) DNA content in a logarithmic population of *sfp1*Δ cells determined by FACS analysis.

result provided further evidence that Sfp1p acts as an inhibitor of progression through G2.

Further analysis of the *SFP1* gene product: We determined the intracellular location of the *SFP1* gene product. To do so, the gene-encoding green-fluorescent protein was fused in-frame to either the 3′ or the 5′ ends of *SFP1.* [In confirmation of our earlier observation that the *SFP1* promoter is weak (Figure 2), we found that the fusion protein needed to be expressed from the *GAL1* promoter for visualization.] Both constructs complemented the slow-growth phenotype of *sfp1*Δ mutants in galactose medium, indicating that the fusion proteins were functional inside cells (data not shown). Fluorescence microscopy demonstrated that the fusion proteins localized with nuclei (Figure 7). Sfp1p therefore appears to be a nuclear protein, consistent with a possible role in transcriptional regulation as predicted from its primary sequence.

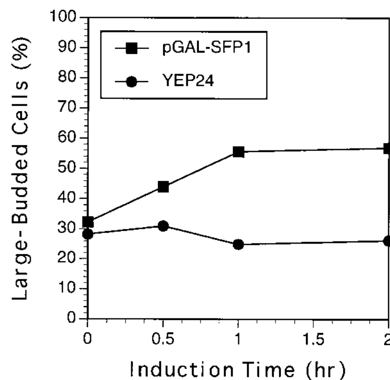

Figure 6.—Overexpression of Sfp1p increases the proportion of large-budded cells. Cultures of wild-type cells carrying YEp24 or pGALSFP1 were grown to early logarithmic phases in 2% raffinose, at which point galactose was added to each to a final concentration of 2%. Aliquots were removed at the designated times after addition of galactose and scored for large-budded cells by microscopy.

Another protein that is known to regulate G2/M transitions in yeast is encoded by the *PDS1* gene (Cohen-Fix *et al.* 1996; Yamamoto *et al.* 1996). We therefore examined whether the expression of *PDS1* is altered in strains that either lack or overexpress Sfp1p. We found that the *PDS1* transcript was expressed at such a low level in wild-type cells and *sfp1*Δ mutants that its visualization by Northern blot analysis was extremely problematic, even after cellular RNAs were preselected by fractionation over oligo(dT) Sepharose (Figure 8). However, when the *SFP1* gene product was expressed at high levels inside cells, the *PDS1* transcript was dramatically induced (Figure 8). Because Pds1p acts as a negative regulator of G2/M transitions (Cohen-Fix *et*

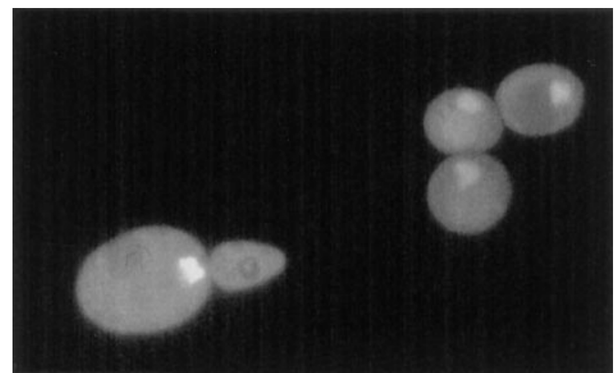

Figure 7.—An Sfp1-GFP fusion protein is localized to the nucleus. A wild-type yeast strain carrying plasmid pSFP-GFP was examined by fluorescence microscopy.

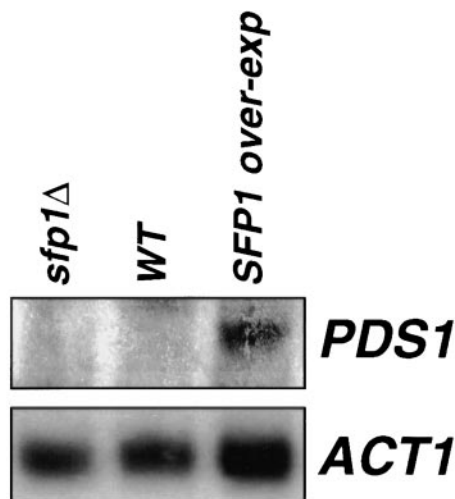

Figure 8.—Overexpression of Sfp1p activates the transcription of *PDS1.* Logarithmic cultures of W303-1A (*WT*), DN1091 (*sfp1*Δ), and DN1093 (*SFP1 over-exp*) were grown in media containing 2% galactose to midlogarithmic phase. Total RNA was prepared from the strains and fractionated over oligo(dT) Sepharose to select poly(A) transcripts. The resulting RNA fractions were subjected to Northern analysis using the *PDS1* and *ACT1* genes as probes.

al. 1996; Yamamoto *et al.* 1996), this may explain why overexpression of Sfp1p delays entrance into mitosis. However, other models are consistent with this observation (see discussion).

DISCUSSION

We have identified a new gene, *SFP1*, whose product is in the DNA-damage checkpoint pathway that blocks cell-cycle progression in late G2. Three experiments demonstrate that Sfp1p plays a critical role in this checkpoint. First, logarithmic cultures of *sfp1*Δ cells do not arrest at the G2/M border after treatment with MMS. Second, resistance to DNA damage is restored to the mutant by artificially imposing a G2 block with nocodazole. Third, the hallmark of checkpoint control—a temporal pause in G2 after DNA damage—fails to occur in the mutant after DNA damage.

The *SFP1* gene product also plays a role in the normal mitotic cell cycle. Our results indicate that Sfp1p acts to repress the transition from G2 into mitosis. This conclusion is based on two results. First, the *sfp1*Δ mutant enters mitosis prematurely, as determined by budding ratios, FACS analysis, and cell size. Second, overexpression of Sfp1p leads to an increase in the number of large-budded cells, consistent with a delayed entrance into mitosis. Because the *sfp1*Δ mutant exhibits altered G2/M regulation in both the normal mitotic cell cycle and the DNA-damage checkpoint pathway, Sfp1p may play the same role in the two phenomena. This could imply that Sfp1p is a checkpoint effector; *i.e.*, it acts at the intersection between the signal-transduction cascade that mediates G2 checkpoint control and the normal mitotic cell-cycle machinery. Specifically, the G2 checkpoint pathway may activate Sfp1p, thereby delaying the cell's entrance into mitosis until the damage has been repaired. It should be noted, however, that we have been unable to demonstrate a complete G2 arrest in cells that overexpress Sfp1p; we demonstrated only a significant delay. This may indicate that the transition from G2 into mitosis in wild-type cells is mediated by an Sfp1p-dependent pathway and another unknown and partially redundant pathway. Alternatively, Sfp1p may be in the only pathway necessary for this transition, but unknown post-translational modifications modulate its activity in the mutant, even when overexpressed. Post-translational modifications have been shown to correlate with arrest at the G2 checkpoint (Navas *et al.* 1996; Sanchez *et al.* 1996; Sun *et al.* 1996).

We currently propose that Sfp1p is involved in transcriptional regulation. This is consistent with two features of the Sfp1p polypeptide: first, it contains zinc-finger domains that are homologous to zinc fingers in known transcription factors (Figure 1); and second, it contains a poly(A)sp sequence, a domain that is often found in yeast transcription factors (Hope and Struhl 1986; Ma and Ptashne 1987). If true, the structure of Sfp1p is somewhat unusual in that its zinc fingers are separated by 37 amino acids, rather than the 7–8 amino acids normally found in transcription factors with canonical Cys_2His_2 zinc fingers (Evans and Hollenberg 1988). A split-zinc-finger motif *per se*, however, does not rule out a role in transcription—two other split-zinc-finger proteins from Drosophila, Teashirt and Su-var(3)7, have been proposed to regulate gene expression (Reuter *et al.* 1990; Fasano *et al.* 1991).

Other observations are consistent with a transcriptional role for the *SFP1* gene product. First, we showed that the polypeptide is localized to the nucleus, as expected for a transcription factor. Second, we isolated two high-copy suppressors of the *sfp1*Δ mutant and identified one of them as *HAP5*, which encodes a component of the CCAAT-binding transcription factor (McNabb *et al.* 1995) (Z. Xu and D. Norris, unpublished results). Finally, overexpression of Sfp1p results in the transcriptional induction of *PDS1*, a known regulator of the G2/M transition, but not *ACT1*, a gene-encoding yeast actin. Sfp1p may therefore act, directly or indirectly, to regulate the transcription of genes like *PDS1*, which are involved in cell-cycle regulation. At this time, however, we cannot rule out the alternative explanation that overexpression of Sfp1p inhibits cell-cycle progression for some reason unrelated to transcription and that the resulting pause at the G2/M border led to the observed activation of *PDS1.*

The Sfp1p polypeptide shares strong homology around its zinc-finger motifs with the *MSN2* gene product of *S. cerevisiae.* Msn2p and the structurally homologous Msn4p are required for the multistress response,

which activates transcription of a large number of genes in response to a diverse array of cellular stresses, including heat shock, DNA alkylation, osmotic shock, oxidative damage, heavy-metal exposure, and nutrient limitations (Estruch and Carlson 1993; Martinez-Pastor *et al.* 1996; Schmitt and McEntee 1996). The Msn2 and Msn4 proteins have been shown to bind to stress-response elements in the promoters of regulated genes and act as positive transcription factors (Martinez-Pastor *et al.* 1996; Schmitt and McEntee 1996). To date, no evidence that *msn2*Δ and *msn4*Δ mutants exhibit cell-cycle defects has been presented. It is intriguing to speculate that stress signals, like DNA alkylation, might activate multiple transcription factors such as Msn2/Msn4, which activate the transcription of stress-inducible genes, and Sfp1p, which activates or represses genes whose products mediate the appropriate cell-cycle response(s).

The *SFP1* gene was originally identified on the basis of its ability, when present on high-copy-number plasmids, to partially block the localization of nuclear proteins (Blumberg and Silver 1991). *sfp1*Δ mutants, however, exhibited normal localization patterns (Blumberg and Silver 1991). This last observation suggests that the role of Sfp1p in the G2/M transition is independent of its role in nuclear localization. However, since the localization patterns of only a limited number of proteins have been analyzed in the *sfp1*Δ mutant, it remains possible that Sfp1p might directly or indirectly affect the localization of some unknown protein(s) that regulates progression into mitosis. It is interesting to note that in human cells, release from radiation-induced arrest in G2 has been proposed to occur as a result of the relocalization of Cyclin B-Cdc2 complexes from the cytoplasm to the nucleus (Jin *et al.* 1996).

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