

## ***sud1*<sup>+</sup> targets cyclin-dependent kinase-phosphorylated Cdc18 and Rum1 proteins for degradation and stops unwanted diploidization in fission yeast**

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**ABSTRACT** In the fission yeast *Schizosaccharomyces pombe*, S phase is limited to a single round per cell cycle through cyclin-dependent kinase phosphorylation of critical replication factors, including the Cdc18 replication initiator protein. Because defects in Cdc18 phosphorylation lead to a hyperstable and hyperactive form of Cdc18 that promotes high levels of overreplication *in vivo*, we wished to identify the components of the Cdc18 proteolysis pathway in fission yeast. In this paper we describe one such component, encoded by the *sud1*<sup>+</sup> gene. *sud1*<sup>+</sup> shares homology with the budding yeast *CDC4* gene and is required to prevent spontaneous re-replication in fission yeast. Cells lacking *sud1*<sup>+</sup> accumulate high levels of Cdc18 and the CDK inhibitor Rum1, because they cannot degrade these two key cell cycle regulators. Through genetic analysis we show that hyperaccumulation of Rum1 contributes to re-replication in  $\Delta$ *sud1* cells, but is not the cause of the defect in Cdc18 proteolysis. Rather, Sud1 itself is associated with the ubiquitin pathway in fission yeast and binds to Cdc18 *in vivo*. Most importantly, Sud1-Cdc18 binding requires prior phosphorylation of the Cdc18 polypeptide at CDK consensus sites. These results provide a biochemical mechanism for the phosphorylation-dependent degradation of Cdc18 and other cell cycle regulators, including Rum1. Evolutionary conservation of the Sud1/CDC4 pathway suggests that phosphorylation-coupled proteolysis may be a general feature of nearly all eukaryotic cell cycles.

The timely and rapid destruction of regulatory proteins is one of the major themes in cell cycle control. In particular, the degradation of cyclins and cyclin-dependent kinase (CDK) inhibitors plays an important role in controlling progression through S phase and mitosis (reviewed in ref. 1). Studies in yeast, *Xenopus* oocytes, and mammalian cells indicate that two distinct proteolytic pathways are important for these cell-cycle events. Degradation of mitotic cyclins at the metaphase-anaphase transition depends on a large ubiquitin-protein ligase (E3) complex, termed the cyclosome or anaphase-promoting complex. Anaphase-promoting complex activity is cell cycle-regulated and uses as its substrate recognition signal a conserved "mitotic destruction box" that is present both in mitotic cyclins and in various noncyclin substrates (1). By contrast, ubiquitination and degradation of G<sub>1</sub> cyclins and other G<sub>1</sub>/S substrates appears to be regulated by the phosphorylation state of the substrate protein. For example, phosphorylation of the budding yeast G<sub>1</sub> cyclin CLN2 and S phase CDK inhibitor SIC1 targets these proteins for rapid degradation by another putative E3 complex, containing the CDC4, CDC34, and CDC53 proteins (2–6).

In fission yeast, genetic analysis has provided insight into the cell cycle controls that prevent excess DNA replication (7, 8). To date, three broad classes of overreplication mutants have been described. The first group comprises mutants in which the activity of mitotic cyclin/CDK complexes (Cdc13/Cdc2 in fission yeast) has been quantitatively eliminated, for example through depletion of the mitotic cyclin Cdc13, or overproduction of the mitotic cyclin/CDK inhibitor Rum1 (9–13). The second group of overreplication mutants consists of those expressing high levels of Cdc18, a key replication initiator protein (6, 14, 15). Interestingly, both Cdc18 and Rum1 are highly unstable proteins that normally accumulate during the G<sub>1</sub> phase of the cell cycle and then are rapidly degraded (13, 14). A third group of overreplication mutants includes the recently described *pop1* mutant (16). Interestingly, fission yeast cells lacking *pop1*<sup>+</sup> were found to express higher than normal levels of Rum1 and Cdc18, although the precise mechanism of this effect (e.g., increased transcription of *cdc18*<sup>+</sup>, increased translation of *cdc18*<sup>+</sup> mRNAs, or reduced degradation of the Cdc18 polypeptide) remains unknown. Taken together, these data suggest that rapid proteolysis of Cdc18 and/or Rum1 might be important in limiting DNA replication to once per cell cycle.

Work in our laboratory has elucidated several genetic and biochemical connections between Cdc18 and CDK activity in fission yeast. (i) Previously we found that increased levels of the Rum1 CDK inhibitor reversed the DNA replication and cell cycle defects of a temperature-sensitive *cdc18* mutant and promoted the stable accumulation of Cdc18 protein during Rum1-induced overreplication (12). (ii) We subsequently reported that fission yeast Cdc18 protein copurifies with S phase and mitotic cyclin/CDK complexes and is a substrate for these kinases *in vitro* (17). (iii) We have recently demonstrated that CDK-dependent phosphorylation of Cdc18 directly inhibits its replication activity and promotes its rapid destruction *in vivo* (6).

In this paper we provide direct evidence that proteolysis of Cdc18 requires the ubiquitin-proteasome pathway. We also describe one of the cellular factors responsible for the phosphorylation-dependent degradation of Cdc18, which we have named *sud1*<sup>+</sup> (stops unwanted diploidization). *sud1*<sup>+</sup> is phylogenetically related to the budding yeast *CDC4* gene and the fission yeast *pop1*<sup>+</sup> gene (16). As its name suggests, cells lacking *sud1*<sup>+</sup> undergo spontaneous re-replication of the genome, resulting in the accumulation of diploid and tetraploid cells. At a molecular level,  $\Delta$ *sud1* mutants are unable to degrade Cdc18 and Rum1 rapidly and accumulate high levels of both cell-cycle proteins. Genetic analysis indicates that these proteolysis defects are independent of one another and sug-

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Abbreviations: CDK, cyclin-dependent kinase; HA, hemagglutinin. Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF064515).

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gests that excessive accumulation of Rum1 in particular plays a major role in spontaneous diploidization. Coimmunoprecipitation experiments demonstrate that Sud1 physically associates both with the ubiquitin pathway and with Cdc18, one of its physiologic substrates. Most strikingly, Sud1 binding requires CDK phosphorylation of Cdc18, thus explaining the phosphorylation dependence of Cdc18 degradation.

## MATERIALS AND METHODS

**Genetic Methods.** Protocols for growth and genetic manipulation of fission yeast have been described (18). The *sud1*<sup>+</sup> gene was identified in a TBLASTN search with the *Saccharomyces cerevisiae* CDC4 protein sequence against the *S. pombe* genome sequence database ([http://www.sanger.ac.uk/Projects/S.pombe/blast\\_server.shtml](http://www.sanger.ac.uk/Projects/S.pombe/blast_server.shtml)). The *sud1*<sup>+</sup> gene physically maps to chromosome I, cosmid c4D7. To delete *sud1*<sup>+</sup> by homologous recombination, sequences 5' and 3' to the *sud1*<sup>+</sup> ORF were separately amplified by PCR, digested with *Xho*I/*Bgl*II and *Bgl*II/*Not*I, respectively, and ligated to *Xho*I/*Not*I-digested pBluescript. This plasmid was linearized with *Bgl*II, treated with calf intestinal alkaline phosphatase, and ligated with a *Bgl*II fragment containing the *his3*<sup>+</sup> gene (19, 20). An *Apa*I/*Not*I fragment of the final disruption construct was transformed into an appropriate diploid strain (*h*<sup>+</sup>/*h*<sup>-</sup> *his3-D1/his3-D1 leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-M210/ade6-M216*). Stable His<sup>+</sup> Ade<sup>+</sup> transformants were isolated and sporulated, and tetrads dissected. Tetrads yielding a monogenic segregation pattern of 2 His<sup>+</sup>:2 His<sup>-</sup> colonies were then analyzed by Southern blotting to verify proper disruption of *sud1*<sup>+</sup>. Because  $\Delta$ *sud1* mutants were sterile, genetic crosses were performed by using a  $\Delta$ *sud1* strain that harbors a *sud1*<sup>+</sup>/*ura4*<sup>+</sup> plasmid. The resulting progeny strains were then tested on Edinburgh minimal medium minus uracil plates to confirm meiotic loss of the plasmid. Cellular DNA content was measured by propidium iodide staining of ethanol-fixed cells and flow cytometry (6).

***nmt1* Promoter Shutoff Experiments.** Acute repression of genes under the control of the thiamine-repressible *nmt1* promoter was achieved by adding thiamine (10  $\mu$ g/ml final) to cells grown overnight in thiamine-free Edinburgh minimal medium. In some experiments, cycloheximide (100  $\mu$ g/ml final) was added at the same time to inhibit ongoing protein synthesis (6). Samples were harvested at the indicated intervals and quick-frozen at -70°C.

**RNA and Protein Analysis.** Procedures for RNA isolation and Northern blot analysis have been described (12). To extract protein from *S. pombe*, two methods were used. For samples to be subjected only to SDS/PAGE, 10<sup>8</sup> cells were lysed with glass beads directly in SDS loading buffer. For soluble native extracts, about 10<sup>9</sup> cells were lysed in ice-cold HB buffer (18), and the supernatant harvested after centrifugation at 15,000  $\times$  *g* for 30 minutes at 4°C. Subsequent immunoprecipitation and immunoblotting procedures were identical to those already described (6, 12). Anti-tubulin mAb B512 and polyclonal anti-ubiquitin antibodies were obtained from Sigma. Anti-HA mAb 12CA5 and anti-MYC mAb 9E10 were purchased from Babco. 9E10 mAb-horseradish peroxidase conjugate was purchased from Boehringer Mannheim. Anti-Rum1 antiserum was a generous gift of Sergio Moreno (Universidad de Salamanca) and was affinity purified before use.

## RESULTS

**Destruction of the Cdc18 Replication Initiator Protein is Mediated by the Ubiquitin-Proteasome Pathway.** To address the mechanism of Cdc18 proteolysis, we examined the role of proteasome function in this process. In fission yeast, the *mts2*<sup>+</sup> and *mts3*<sup>+</sup> gene products have been identified as subunits of

the 26S proteasome, which is responsible for the ubiquitin-dependent degradation of a variety of cellular proteins in eukaryotic cells (21, 22). We used temperature-sensitive mutations in *mts2*<sup>+</sup> and *mts3*<sup>+</sup> to inactivate proteasome function conditionally. Increased expression of Cdc18 in *mts3-1* (but not *mts2-1*) mutants has been reported (16), although it is not clear whether this reflects an increased half-life of the Cdc18 polypeptide, or merely accumulation of cells in a portion of the cell cycle where *cdc18*<sup>+</sup> is highly transcribed. To circumvent these limitations in our experiments, Cdc18 was expressed constitutively from an attenuated version of the thiamine-repressible *nmt1* promoter. We found that Cdc18 readily accumulated in both *mts2-1* and *mts3-1* strains at 36°C (Fig. 1A). We also observed a ladder of higher molecular weight species, consistent with the accumulation of Cdc18-multiubiquitin adducts in the absence of proteasome function. To demonstrate directly that Cdc18 proteolysis requires the proteasome, we acutely repressed Cdc18 synthesis from the *nmt1* promoter with thiamine and monitored protein turnover by immunoblotting (Fig. 1B). As expected, Cdc18 is rapidly destroyed in wild-type cells, with a half-life of about 5 min (6, 14). By contrast, Cdc18 was degraded very slowly in *mts2-1* and *mts3-1* mutant cells, persisting for up to 1 hr after shutoff (Fig. 1B). Taken together, our data indicate that Cdc18 proteolysis requires the ubiquitin-proteasome pathway.

**Identification of *sud1*<sup>+</sup>, a CDC4-Related Gene in Fission Yeast.** We next examined a number of *S. pombe* mutants for their effects on Cdc18 degradation. We observed that the anaphase-promoting complex was probably not responsible for the bulk of Cdc18 proteolysis, as the half-life of Cdc18 in *nuc2-663* and *cut9-665* mutants was similar to that in wild-type (data not shown). This result confirms that the stabilization of Cdc18 in proteasome-defective cells (Fig. 1) was not an indirect effect of cell cycle synchronization, as *mts2-1*, *mts3-1*, *nuc2-663*, and *cut9-669* mutants all arrest at the metaphase-anaphase transition with high levels of mitotic CDK activity (21–24). To identify cellular factors participating in Cdc18 proteolysis, we considered the fact that CDK phosphorylation of Cdc18 plays a major role in targeting the polypeptide for destruction (6). Because degradation of the cell-cycle phosphoproteins CLN2 and SIC1 is mediated by the *CDC4*, *CDC34*, and *CDC53* gene products in budding yeast, we searched for fission yeast homologs as potential Cdc18 proteolysis factors. Systematic sequencing of the fission yeast genome has uncovered a gene related to *CDC4*, which we have named *sud1*<sup>+</sup>. The *sud1*<sup>+</sup> gene encodes a polypeptide of 704 amino acids that is 32% identical to the budding yeast CDC4 protein and 35% identical to the *pop1*<sup>+</sup> gene product. The maximum homology between these proteins is located within the C-terminal WD-40 repeats, while the N-terminal regions are much less well conserved. The N terminus of Sud1 contains a recognizable F-box sequence motif (LPFSIVQSILLNLDIHSFLSCRLVSPWNRILDVHTSYWKHM; amino acids 242–283). This motif appears to mediate physical interaction of CDC4 and other F-box proteins with SKP1 (25).

***sud1*<sup>+</sup> Stops Unwanted Diploidization.** To analyze the function of *sud1*<sup>+</sup>, we constructed a fission yeast strain in which the *sud1*<sup>+</sup> gene was deleted, having been replaced by the *his3*<sup>+</sup> prototrophic marker. After sporulation of a heterozygous  $\Delta$ *sud1/sud1*<sup>+</sup> diploid strain, we readily recovered tetrads in which two of the colonies were His<sup>+</sup> and two were His<sup>-</sup>, indicating that *sud1*<sup>+</sup> is not essential for viability. However, the  $\Delta$ *sud1* cells were frequently larger than wild-type cells and gave rise to darkly staining colonies on media containing the vital dye phloxin B. Because similar attributes are observed with diploid fission yeast cells, we examined the genomic DNA content of  $\Delta$ *sud1* cells by flow cytometry. Interestingly, the  $\Delta$ *sud1* mutant frequently carried out an extra round of DNA replication without mitosis, resulting in the accumulation of cells with diploid (4C) DNA content (Fig. 2A). We conclude

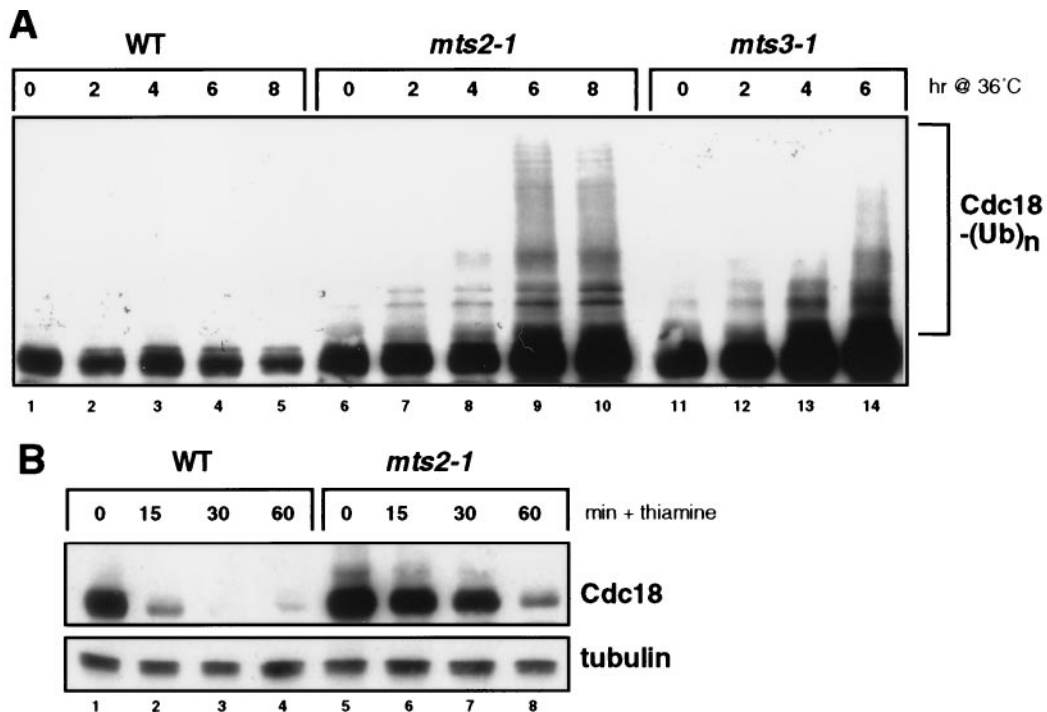


FIG. 1. Degradation of the Cdc18 replication initiator protein is mediated by the ubiquitin-proteasome pathway in fission yeast. (A) A hemagglutinin (HA) epitope-tagged form of Cdc18 was expressed from the weak thiamine-repressible *nmt1* promoter (REP41X) in a wild-type strain (lanes 1–5) or in two temperature-sensitive (ts) proteasome mutants (*mts2-1*, lanes 6–10; *mts3-1*, lanes 11–14). Logarithmically growing cells were shifted from 25°C to 36°C, and the abundance and electrophoretic mobility of Cdc18 assessed by immunoblotting with anti-HA mAb 12CA5. The position of the Cdc18-multiubiquitin adducts is indicated. (B) The half-life of Cdc18 in wild-type and *mts2-1* strains was measured by shifting cells to 36°C for 3 hr (to inactivate the mutant proteasomes) and then adding thiamine to acutely repress *de novo* expression from the *nmt1* promoter. Cdc18 degradation was monitored by immunoblotting with 12CA5 mAb. The same membrane was blotted with anti-tubulin mAb B512 to confirm equal loading.

that the normal function of *sud1*<sup>+</sup> is to suppress unwanted diploidization, thus ensuring stable maintenance of the haploid state during vegetative growth. This may account in part for the fact that deletion of *sud1*<sup>+</sup> also results in sterility (data not shown).

**Rapid Proteolysis of Cdc18 and Rum1 Requires *sud1*<sup>+</sup>.** To determine the molecular basis of this re-replication phenotype, we examined the levels of several important cell-cycle regulatory proteins in  $\Delta$ *sud1* cells by immunoblotting. We found that both the Rum1 CDK inhibitor and Cdc18 replication initiator proteins accumulated to abnormally high levels in the  $\Delta$ *sud1* mutant strain (Fig. 2B). Interestingly, the increased abundance of Cdc18 and Rum1 was not a consequence of increased gene transcription or mRNA stability, as the abundance of *cdc18*<sup>+</sup> and *rum1*<sup>+</sup> transcripts was the same in  $\Delta$ *sud1* and wild-type strains (Fig. 2C).

Formally, the accumulation of Cdc18 and Rum1 in  $\Delta$ *sud1* cells could reflect either increased translation of the corresponding mRNAs or decreased degradation of the two polypeptides. To distinguish these two possibilities, HA epitope-tagged forms of *cdc18*<sup>+</sup> and *rum1*<sup>+</sup> were expressed from the weak *nmt1* promoter, and the turnover of Cdc18 and Rum1 was monitored after adding thiamine and cycloheximide simultaneously to repress transcription and *de novo* translation (Fig. 2D). While Cdc18 and Rum1 were degraded quickly in wild-type cells, both proteins persisted in  $\Delta$ *sud1* cells for more than one hour after shutoff. These data demonstrate that *sud1*<sup>+</sup> is required for the rapid proteolysis of both Cdc18 and Rum1 proteins *in vivo*.

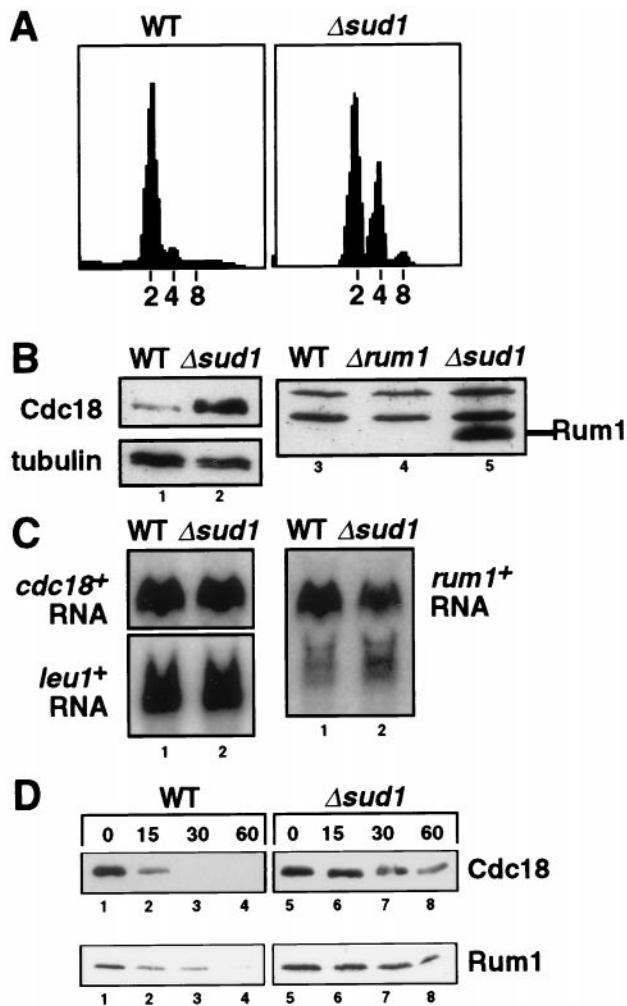
Because increased Rum1 expression itself can increase Cdc18 abundance (12), it was of interest to determine if Rum1 was required for the stable accumulation of Cdc18 in the  $\Delta$ *sud1* mutant. A  $\Delta$ *sud1*  $\Delta$ *rum1* double mutant strain was constructed, and Cdc18 proteolysis examined as in the previous experiment.

Again, Cdc18 proteolysis was just as defective in the  $\Delta$ *sud1*  $\Delta$ *rum1* mutant (Fig. 3A). We conclude that the requirement for *sud1*<sup>+</sup> in Cdc18 degradation is not an indirect effect of increased Rum1 stability.

**Rum1 Stabilization Plays a Major Role in the  $\Delta$ *sud1* Re-replication Phenotype.** The  $\Delta$ *sud1*  $\Delta$ *rum1* double mutant also allowed us to test the role of Rum1 stability in the re-replication phenotype caused by loss of *sud1*<sup>+</sup>. Wild-type,  $\Delta$ *sud1*, and  $\Delta$ *sud1*  $\Delta$ *rum1* strains were grown overnight in minimal medium containing low nitrogen (0.25% NH<sub>4</sub>Cl). This treatment produces high levels of re-replication in the  $\Delta$ *sud1* strain, as shown by the appearance of 4C and tetraploid 8C cells (Fig. 3B). By contrast, the  $\Delta$ *sud1*  $\Delta$ *rum1* mutant strain largely retained the normal 2C DNA content. These data suggest that Rum1 stabilization plays a major role in the re-replication cycles of the  $\Delta$ *sud1* mutant strain, although we have not excluded the possibility that increased Cdc18 abundance also contributes to the re-replication phenotype.

**Characterization of the Sud1 Protein.** To address the biochemical role of Sud1 in Cdc18 and Rum1 proteolysis, we examined the properties of the Sud1 protein itself. For these studies, an HA-tagged form of Sud1 was expressed in fission yeast. This protein was shown to be functional, as it complemented the sterility and diploidization phenotypes of the  $\Delta$ *sud1* mutant (data not shown) and was readily detected with anti-HA monoclonal antibody 12CA5.

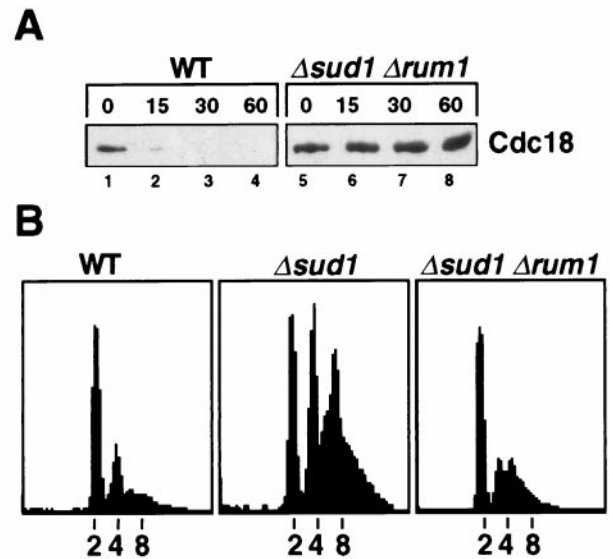
We performed immunoprecipitation experiments to determine if Sud1 was associated with ubiquitinated proteins in fission yeast. Extracts were incubated with antibodies to either ubiquitin or HA-Sud1, and the resulting immunoprecipitates immunoblotted with the reciprocal antibody. Interestingly, Sud1 was readily precipitated by ubiquitin-specific antibodies (Fig. 4 Left). Moreover, a ladder of large, ubiquitin-containing species was observed in HA-Sud1 immunoprecipitates, which



**FIG. 2.** *sud1*<sup>+</sup> stops unwanted diploidization and is required for rapid degradation of both Cdc18 and the fission yeast CDK inhibitor Rum1. (A) DNA content of wild-type and  $\Delta sud1$  cells was analyzed by propidium iodide staining and flow cytometry. The positions of normal haploid (2C), diploid (4C), and tetraploid (8C) DNA contents are indicated. (B) The abundance of Cdc18 and Rum1 proteins in wild-type,  $\Delta sud1$ , and  $\Delta rum1$  strains was measured by immunoblotting with specific antibodies. (C) Northern blot analysis of *cdc18*<sup>+</sup> and *rum1*<sup>+</sup> mRNA transcripts in wild-type (lane 1) and  $\Delta sud1$  cells (lane 2). The *leu1*<sup>+</sup> mRNA was used as a loading control. PhosphorImager quantitation demonstrated that *cdc18*<sup>+</sup> and *rum1*<sup>+</sup> mRNAs were equally abundant in both strains. (D) Increased half-life of Rum1 and Cdc18 proteins in  $\Delta sud1$  strains. HA-tagged forms of Cdc18 (Upper) and Rum1 (Lower) were expressed under the control of the weak *nmt1* promoter in either wild-type cells (lanes 1–4) or  $\Delta sud1$  cells (lanes 5–8). Degradation of Cdc18 and Rum1 was monitored after acutely repressing *de novo* synthesis with thiamine and cycloheximide.

represent cellular protein-multiubiquitin adducts (Fig. 4 Right). These data indicate that Sud1 associates with the ubiquitin pathway in fission yeast, most likely through interactions with cellular proteins that are undergoing multiubiquitination and degradation.

To test for associations with specific target proteins, we examined the ability of Sud1 to interact physically with Cdc18. Through coprecipitation studies, we found that Sud1 and Cdc18 bind one another *in vivo* (Fig. 5A). To determine the functional significance of this interaction, we examined the role of Cdc18 phosphorylation in the process. As we reported previously, mutation of the six CDK consensus sites in Cdc18 abolishes its *in vivo* phosphorylation pattern and renders it immune to rapid degradation (6). Interestingly, the Cdc18 $\Delta$ CDK1–6 mutant protein is incapable of binding to

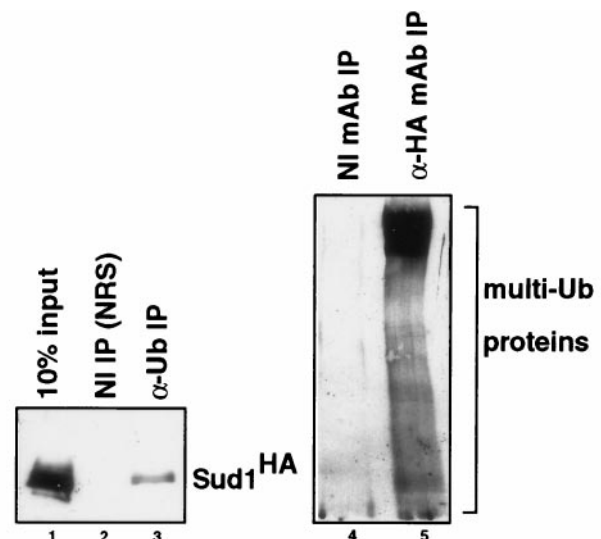


**FIG. 3.** Rum1 stabilization is required for re-replication of the genome in  $\Delta sud1$  cells but does not cause the defect in rapid proteolysis of Cdc18. (A) Degradation of Cdc18 in wild-type and  $\Delta sud1$   $\Delta rum1$  strains was measured as described above (see Materials and Methods). (B) Wild-type,  $\Delta sud1$ , and  $\Delta sud1$   $\Delta rum1$  strains were grown overnight in minimal medium containing low nitrogen to induce re-replication. DNA content was analyzed by flow cytometry.

Sud1, unlike wild-type Cdc18 (Fig. 5B). These data indicate that Sud1 binding requires phosphorylation of Cdc18 by fission yeast CDKs and is required for rapid proteolysis of Cdc18.

## DISCUSSION

Degradation of cyclins and other regulatory proteins plays a major role in promoting cell-cycle progression in eukaryotic cells. As shown both in this paper and a recent report by



**FIG. 4.** Sud1 is physically associated with the ubiquitin pathway in fission yeast. (Left) Extracts containing HA-tagged Sud1 protein were immunoprecipitated with either control nonimmune rabbit serum (lane 2) or anti-ubiquitin antibodies (lane 3), separated by SDS/PAGE, and immunoblotted with anti-HA mAb 12CA5. One-tenth of the original extract was loaded on the same gel for comparison. (Right) Extracts containing HA-tagged Sud1 were immunoprecipitated with either control nonimmune mAb (lane 4) or HA-specific mAb 12CA5 (lane 5), and then immunoblotted with anti-ubiquitin antibodies. The position of high molecular weight protein-multiubiquitin adducts is indicated.

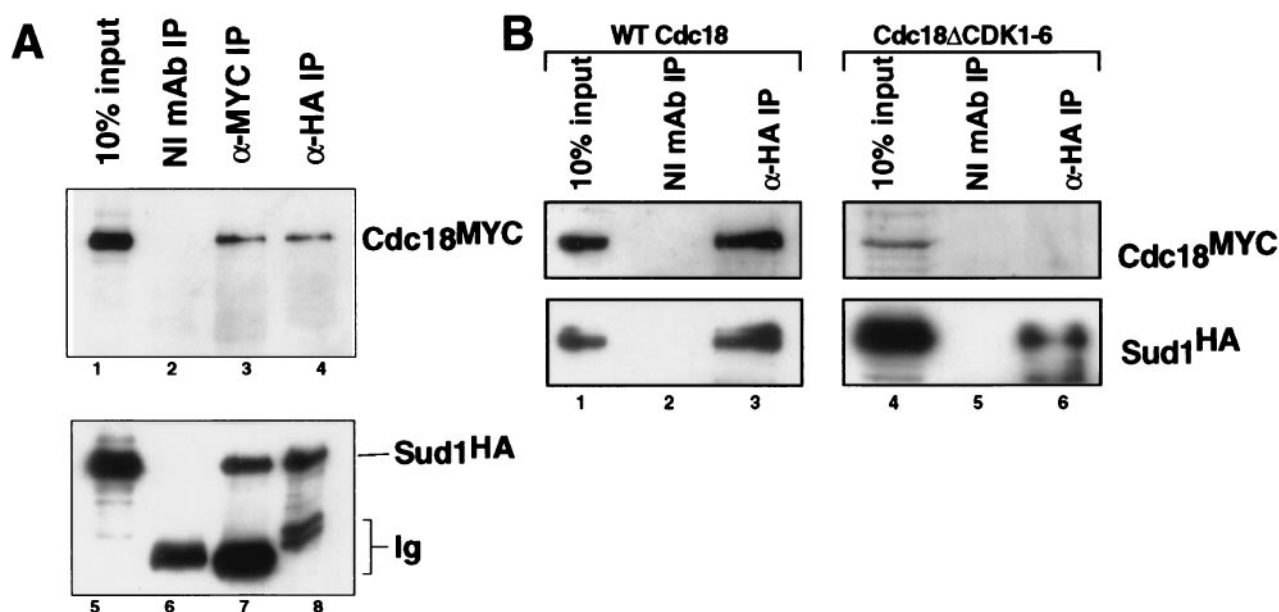


FIG. 5. Sud1 and Cdc18 bind one another in a phosphorylation-dependent manner. (A) Extracts derived from cells expressing HA epitope-tagged Sud1 and MYC epitope-tagged Cdc18 were immunoprecipitated with either control nonimmune mAb (lanes 2 and 6), anti-HA mAb 12CA5 (lanes 3 and 7), or anti-MYC mAb 9E10 (lanes 4 and 8). Immunoprecipitates and an aliquot of the input extract (lanes 1 and 5) were separated by SDS/PAGE and immunoblotted with either 9E10-horseradish peroxidase conjugate (9E10-HRP; *Upper*) or 12CA5 mAb (*Lower*) Ig, immunoglobulin. (B) HA-Sud1 was expressed together with either wild-type MYC-Cdc18 or MYC-Cdc18ΔCDK1-6. Extracts were immunoprecipitated with control nonimmune mAb (lanes 2 and 5) or 12CA5 mAb (lanes 3 and 6), then immunoblotted with 9E10-horseradish peroxidase conjugate (*Upper*) and 12CA5 mAb (*Lower*). Aliquots of the input extracts (lanes 1 and 4) were included on the same gel for comparison.

Kominami and Toda (16), proteolysis can also play an important role in preventing reinitiation of DNA replication without mitosis, which otherwise would lead to abnormal increases in genome ploidy.

Recently we (6) and Benito *et al.* (26) demonstrated that phosphorylation of Cdc18 and Rum1 proteins by Cdc2 kinase (the major CDK in fission yeast) targets both substrates for rapid degradation. In this paper we verified that Cdc18 is degraded by the ubiquitin-proteasome pathway and have identified one of the cellular factors responsible for this process, which is encoded by the fission yeast *sud1*<sup>+</sup> gene. Through genetic analysis we demonstrated that *sud1*<sup>+</sup> stops unwanted diploidization and ensures stable maintenance of normal chromosomal DNA content in fission yeast. *sud1*<sup>+</sup> is related to both *S. cerevisiae* CDC4 and *S. pombe* pop1<sup>+</sup> and contains the F-box and WD-40 repeat motifs characteristic of this gene family (25). The Sud1 protein is required for rapid proteolysis of at least two important cell cycle regulators in fission yeast, namely the Cdc18 and Rum1 proteins (10, 27). Interestingly, we find that Sud1 interacts with ubiquitinated proteins in fission yeast and binds the Cdc18 polypeptide in a phosphorylation-dependent manner. These data suggest an attractive model for how CDK phosphorylation targets Cdc18 for destruction *in vivo*, namely by promoting the physical interaction of Cdc18 with Sud1 and other components of the ubiquitin-proteasome pathway. These additional components have not been identified yet in fission yeast but are likely to include homologs of the CDC34/UBC and CDC53/cullin protein families (25, 28, 29).

The *sud1*<sup>+</sup> and *pop1*<sup>+</sup> genes share many similarities in their biochemical and genetic properties. Cells lacking either gene undergo diploidization at increased frequency and accumulate increased levels of both Cdc18 and Rum1 polypeptides, demonstrating that both *sud1*<sup>+</sup> and *pop1*<sup>+</sup> are necessary to maintain these two critical cell-cycle regulators at physiological levels (16). Both Sud1 and Pop1 proteins bind Cdc18, and at least for Sud1 (but probably for Pop1 as well) this binding requires phosphorylation of the Cdc18 substrate at multiple CDK consensus sites. An important question is why fission

yeast would have evolved two similar genes with apparently overlapping functions. One possibility is that while both Pop1 and Sud1 bind Cdc18 and target it for destruction, there may be other cellular phosphoproteins that are specifically targeted for degradation by Sud1 but not Pop1 (or *vice versa*). Alternatively, Pop1 and Sud1 may not have distinct substrate specificities, but may simply act additively to ensure rapid and quantitative degradation of target molecules.

Interestingly, degradation of CDC6 (the homolog of Cdc18 in budding yeast) also requires CDC4 (30, 31), although the role of CDK phosphorylation in this process has not yet been established. Given the evolutionary conservation of Sud1/Pop1/CDC4 homologs among not only budding and fission yeasts but also multicellular organisms (32–34), it seems likely that there will be additional important targets of this phosphorylation-coupled proteolysis pathway, particularly during developmental and neoplastic modulation of cell cycle progression in higher eukaryotes (32–34).

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