Rum1 and Cdc18 link inhibition of cyclin-dependent kinase to the initiation of DNA replication in *Schizosaccharomyces pombe*

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Eukaryotic cells have evolved regulatory mechanisms to ensure the strict alternation of DNA replication and mitosis. Recent work has suggested that the mitotic form of cyclin-dependent kinase (Cdc2/cyclin B) has a role in preventing re-replication of the genome before mitosis, but the relevant targets of this inhibition are unknown. In this report we present evidence that the mitotic cyclin-dependent kinase affects DNA replication by inhibiting the accumulation and function of Cdc18, a critical regulator of S-phase entry. We found that the *rum1*⁺ gene efficiently suppresses the lethality of a conditional *cdc18* mutant. Conversely, deletion of *rum1*⁺ increases the severity of the *cdc18* mutant phenotype, resulting in inappropriate cell division and a rapid loss of viability. Biochemical experiments indicate that Rum1 potently inhibits Cdc2 phosphorylation of histone H1 or a Cdc18 fusion protein by directly interacting with the Cdc2/cyclin B complex. Overexpression of Rum1 under conditions that promote re-replication of the genome induces a striking accumulation of Cdc18 protein by a largely post-transcriptional mechanism. Overexpression of SIC1, an unrelated cyclin-dependent kinase inhibitor from budding yeast, causes a similar accumulation of Cdc18 and also leads to re-replication. Our data link a potent inhibitor of Cdc2 kinase to a key protein required for the initiation of DNA replication and strongly suggest that inhibition of Cdc18 by cyclin-dependent kinases has an important role in ensuring that the genome is duplicated precisely once each cell cycle.

[Key Words: CDK inhibitor; fission yeast; S phase; cell cycle; re-replication]

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Eukaryotic DNA replication is regulated in the cell cycle to ensure that the genome is duplicated precisely once before mitosis and cell division. Initiation of DNA replication at the beginning of S phase depends on an earlier decision point in G₁ (called Start in yeasts and the "restriction point" in mammalian cells) at which cell size, nutritional status, and extracellular signals are integrated to determine whether or not to begin a new cell cycle (for review, see Forsburg and Nurse 1991; Hunt and Murray 1993). Once S phase is complete, a new round of replication does not occur until passage through mitosis, as shown by the ability of G₁, but not G₂, cells to initiate DNA synthesis when fused with cells already in S phase (Johnson and Rao 1970; Rao and Johnson 1970). Thus, eukaryotic cells actively prevent re-replication in a single cell cycle, presumably by inactivating one or more components of the replication machinery after the beginning of S phase.

A candidate model to explain these observations was

proposed on the basis of in vitro studies of DNA replication in *Xenopus* extracts. In this system, replication of sperm chromatin normally requires passage through mitosis. However, it was discovered that this control could be bypassed by puncturing or otherwise permeabilizing the nuclear membrane, leading to another round of replication (Blow and Laskey 1988). To explain these results, it was suggested that the nuclear membrane prevented an essential replication factor (termed "licensing factor") from associating with chromatin and rendering it competent for replication. Because this factor was inactivated or destroyed during S phase, G₂ nuclei regain replication competence only upon nuclear envelope breakdown at mitosis, at which point licensing factor gains access to the nucleus. Recently, several reports have identified homologs of the minichromosome maintenance (MCM) gene family as proteins required for the replication of G₂ nuclei (Chong et al. 1995; Kubota et al. 1995; Madine et al. 1995;). However, it is unclear whether these MCM homologs constitute the putative licensing factor *Xenopus*. MCM3, for example, is capable of entering the nucleus in the absence of nuclear enve-

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lope breakdown (Madine et al. 1995). In addition, purified MCMs have not yet been shown to be sufficient to trigger the re-replication of permeabilized G₂ nuclei, which is a critical feature of the licensing factor model.

An alternative model for the mechanism governing the proper alternation of DNA replication and mitosis has emerged quite recently. A number of current studies suggest that the mitotic forms of cyclin-dependent kinases (CDKs) have an important role in blocking re-replication before mitosis (Lehner and O'Farrell 1990; Adachi and Laemmli 1994; Hayles et al. 1994; Sauer et al. 1995; for review, see Su et al. 1995). Treatments that inactivate either the CDK or cyclin component of such kinases can result in multiple rounds of DNA replication without an intervening mitosis. For example, fission yeast cells that lack cyclin B because of deletion of the *cdc13⁺* gene amass DNA contents several times normal (Hayles et al. 1994). Similarly, overexpression of Rum1, a putative CDK inhibitor, can also result in re-replication (Moreno and Nurse 1994). These data strongly suggest that the presence of active Cdc2/cyclin B kinase inhibits initiation of DNA replication, but the relevant targets of this inhibition are unknown.

In fission yeast, entry into S phase is dependent on the activation at Start of a cell cycle-regulated transcription factor containing the products of the *cdc10⁺* and *sct1⁺* genes (Lowndes et al. 1992; Caligiuri and Beach 1993). A major target of the Cdc10/Sct1 transcriptional activator is the *cdc18⁺* gene, which has a central role in the regulation of DNA replication. Genetic analysis indicates that *cdc18⁺* is absolutely required for initiation of DNA synthesis and for the checkpoint mechanism that prevents mitosis when DNA synthesis is blocked (Kelly et al. 1993). Analysis of a conditional allele of *cdc18⁺* indicates that the *cdc18⁺* gene product functions within a brief interval of G₁ after traversal of Start but before the hydroxyurea-sensitive point in early S phase (Nasmyth and Nurse 1981). Recent studies demonstrate that Cdc18 is an extremely labile protein that is synthesized de novo once each cell cycle just before the onset of S phase and then disappears rapidly (Nishitani and Nurse 1995; Muzi-Falconi et al. 1996). Cdc18 is related to the CDC6 protein of budding yeast, which is also expressed periodically and functions to promote DNA replication (Liang et al. 1995; Piatti et al. 1995).

In this report we present evidence that the mitotic cyclin-dependent kinase prevents re-replication at least in part by blocking the accumulation of active Cdc18 protein. This connection between the fundamental cell cycle engine and the replication machinery was discovered during a search for genes that rescued the lethality of a temperature-sensitive mutant of *cdc18* (*cdc18-K46*) when present on a multicopy plasmid. The suppressor of *cdc18-K46* isolated in this genetic screen proved to be the *rum1⁺* gene, which has been suggested to encode an inhibitor of cyclin-dependent kinases. Further genetic analysis revealed that deletion of *rum1⁺* specifically accentuates the phenotype of the *cdc18-K46* allele, causing a rapid loss of viability and abnormal cell division. A Rum1 fusion protein inhibited the kinase activity of fis-

sion yeast Cdc2/cyclin B on histone H1 and Cdc18 fusion protein substrates in vitro through physical interaction with the enzyme complex. When Rum1 was overexpressed under the control of a strong promoter, we observed a large increase in Cdc18 protein that was temporally correlated with re-replication of the genome. The accumulation of Cdc18 under these conditions was mediated by a largely post-transcriptional mechanism. These phenomena apparently resulted from the loss of Cdc2 kinase activity because overexpression of SIC1, an unrelated CDK inhibitor from budding yeast, yielded a similar accumulation of Cdc18 and also induced re-replication. Taken together with the recent observation that overproduction of Cdc18 itself can induce re-replication (Nishitani and Nurse 1995; Muzi-Falconi et al. 1996), our findings suggest that inhibition of Cdc18 by CDKs has an important role in ensuring that the genome is duplicated precisely once each cell cycle.

Results

Isolation of the rum1⁺ gene as a suppressor of cdc18-K46 temperature sensitivity

The *cdc18⁺* gene product has a central role in coupling DNA replication to cell cycle commitment at Start as well as to mitosis and cell division. To understand how *cdc18⁺* might be involved in coordinating these cell cycle transitions, we sought to identify new genes that interact functionally with *cdc18⁺* and thus may participate in the same genetic pathways. We carried out a screen to isolate genes that can suppress the temperature-sensitive growth defect of a conditional *cdc18* mutant (*cdc18-K46*). Incubation of this mutant at the restrictive temperature of 36°C results in defective chromosomal replication and cell cycle arrest before mitosis and cell division, producing the characteristic *cdc* phenotype of an elongated cell with a single nucleus (Nasmyth and Nurse 1981; Kelly et al. 1993).

A strain carrying the *cdc18-K46* mutation (PJ18) was transformed with a genomic DNA library constructed in the multicopy plasmid pON163. Among ~50,000 Ura⁺ transformants obtained at 25°C, 16 transformants were isolated that were capable of growth at 36°C. Plasmids were recovered from these colonies, and upon retransformation each could restore viability to the *cdc18-K46* strain at the restrictive temperature. Restriction digestion and Southern hybridization showed that the rescuing clones fell into two distinct groups. Eleven isolates with the strongest suppression phenotype each harbored the *cdc18⁺* gene, as was expected. The remaining five plasmids appeared to suppress less strongly, requiring ~5–6 days to form colonies at 36°C (~1 day longer than for wild type). These plasmids contained overlapping inserts distinct from the *cdc18⁺* gene. Restriction mapping and unidirectional deletion analysis of these inserts delineated a 2.5-kb genomic fragment that was necessary and sufficient for suppression.

Sequencing of this minimal suppressing fragment re-

vealed that the cloned *cdc18-K46* suppressor was identical to the *rum1*⁺ gene (Fig. 1). This finding was of interest because the *rum1*⁺ gene was described previously as a fission yeast complementary DNA (cDNA) clone that induces multiple rounds of DNA replication without mitosis when overexpressed to high levels (Moreno and Nurse 1994). *rum1*⁺ has an important role in progression through the early G₁ interval, with genetic properties suggestive of an inhibitor of Cdc2 kinase (Moreno et al. 1994; Nurse 1994; Peter and Herskowitz 1994b).

Suppression of the *cdc18* mutant phenotype by the *rum1*⁺ genomic plasmid was incomplete at 36°C, because the suppressed cells were still elongated relative to wild type. Flow cytometry of these cells demonstrated a normal 2C DNA content, indicating that they were delayed in the G₂/M interval. This delay is apparently a residual effect of the *cdc18-K46* mutation, because the *rum1*⁺ genomic plasmid did not perturb growth or cell cycle progression when introduced into a wild-type *Schizosaccharomyces pombe* strain (data not shown). To determine whether *rum1*⁺ could rescue the *cdc18-K46*

defect when present in a single extra copy, a plasmid containing the *rum1*⁺ suppressor was introduced into PJ18 and integrated into the genome, yielding a strain with one copy of the *cdc18-K46* mutation and two tandem copies of the *rum1*⁺ gene. The resulting *rum1*⁺ integrant strain exhibited growth at 36°C comparable to that observed with the multicopy *rum1*⁺ plasmid (Fig. 1A). Thus, the *cdc18-K46* temperature-sensitive phenotype is exquisitely sensitive to *rum1*⁺ dosage and can be suppressed by even a single extra copy of the *rum1*⁺ gene.

However, *rum1*⁺ was not capable of rescuing a null allele of *cdc18*⁺ even when present in multiple copies, as demonstrated by the following experiment. A strain in which the *cdc18*⁺ promoter has been substituted by a modified version of the thiamine-repressible *nmt1* promoter was constructed by gene replacement (strain YMF15; A. Russo and T.J. Kelly, unpubl.). Incubation of this strain in medium containing thiamine abolishes transcription of *cdc18*⁺ and leads to mitosis in the absence of DNA replication, as described previously for a complete deletion of the *cdc18*⁺ gene (Kelly et al. 1993).

This *cdc18*-shutoff strain was transformed with genomic plasmids containing either *cdc18*⁺ or *rum1*⁺ and plated on selective medium lacking thiamine. The resulting transformants were then replica-plated onto medium containing thiamine. Cells containing the *cdc18*⁺ genomic plasmid continued to grow normally, but those containing the *rum1*⁺ genomic plasmid failed to grow in the presence of thiamine and appeared to have undergone cell division without DNA replication (data not shown).

We conclude that *rum1*⁺ readily suppresses *cdc18*-*K46* temperature sensitivity, even when present in a single extra copy, but cannot replace the essential function of the *cdc18*⁺ gene. These results suggest that *cdc18*⁺ and *rum1*⁺ functionally interact along a common genetic pathway *in vivo*.

Deletion of rum1⁺ increases the severity of the cdc18-K46 defect and eliminates mitotic restraint

To characterize the genetic interaction between *cdc18*⁺ and *rum1*⁺ further, we examined the consequences of deleting *rum1*⁺ from cells carrying the *cdc18*-*K46* mutation. We constructed a strain harboring the *cdc18*-*K46* mutation and a deletion of *rum1*⁺ (PJ109); at 25°C this

strain exhibited morphology and growth kinetics comparable to the parental *cdc18*-*K46* mutant. The *cdc18*-*K46* $\Delta rum1$ double mutant and isogenic wild-type, $\Delta rum1$, and *cdc18*-*K46* strains were grown to mid-exponential phase at 25°C and then shifted rapidly to 36°C to inactivate the *cdc18*-*K46* allele.

Both wild-type and $\Delta rum1$ strains grew with normal morphology and kinetics, as expected (Fig. 2A,B). The *cdc18*-*K46* strain arrested as an elongated cell with a single nucleus and maintained a high degree of viability during prolonged incubation at 36°C (Fig. 2C,H). In sharp contrast, the *cdc18*-*K46* $\Delta rum1$ double mutant strain continued to divide in the presence of the *cdc18*-*K46* defect. This new round of cell division results in the conspicuous appearance of cells either lacking nuclei altogether or having nuclear material improperly divided between the two halves of a newly septated cell (Fig. 2D-G).

This terminal morphology is often referred to as the *cut* phenotype (Hirano et al. 1986) and reflects loss of the checkpoint mechanism that restrains mitosis and cell division when DNA replication is incomplete (Enoch and Nurse 1990; Kelly et al. 1993). By the end of the temperature-shift experiment, nearly 30% of the *cdc18*-*K46* $\Delta rum1$ cells had executed an inappropriate cell division event, giving rise to the *cut* phenotype. This mor-

Figure 2. Deletion of *rum1*⁺ increases the severity of the *cdc18*-*K46* defect, resulting in inappropriate cell division and a rapid loss of viability. Micrographs of wild-type (A), $\Delta rum1$ (B), *cdc18*-*K46* (C), and *cdc18*-*K46* $\Delta rum1$ strains (D-G) after shifting from 25°C to 36°C for 8 hr. Examples of anucleate and aberrantly septated cells (cuts) are indicated by arrows. (H) Percent abnormal cell division and cell viability in wild-type (TK8; □), $\Delta rum1$ (PJ101; △), *cdc18*-*K46* (PJ18; ●), and *cdc18*-*K46* $\Delta rum1$ strains (PJ109; ▲) during incubation at 36°C. Abnormal cell division was determined from microscopic examination of at least 200 cells from each sample. Cell viability was determined by plating uniform dilutions of each culture onto rich medium and counting colonies after 5 days at 25°C. Percent cell viability is calculated relative to control samples taken immediately before the temperature shift. Similar results were obtained in several independent experiments.

phological measurement underestimates the true frequency of lethal events in this population, as >97% of *cdc18-K46 Δrum1* cells became inviable after 4 hr at 36°C (Fig. 2H).

Several lines of genetic evidence argue that the increased lethality and inappropriate cell division observed in the *cdc18-K46 Δrum1* double mutant represent a specific synthetic interaction. First, the *Δrum1* mutant itself is not accelerated towards mitosis during normal vegetative growth; rather, it divides at the same size and with the same generation time as a wild-type strain (Fig. 2; Moreno and Nurse 1994). Second, *rum1*⁺ is not required for cell cycle arrest in response to the DNA synthesis inhibitor hydroxyurea (Moreno and Nurse 1994). Third, deletion of *rum1*⁺ does not affect cell cycle arrest resulting from temperature-sensitive mutations in several other genes involved in DNA replication, including *cdc17* (DNA ligase), *cdc20* (unknown G₁/S-phase function) and *cdc22* (ribonucleotide reductase; P.V. Jallepalli and T.J. Kelly, unpubl.). Interestingly, loss of *cdc10* function, a condition that abolishes transcription of *cdc18*⁺ at Start (Kelly et al. 1993), has also been reported to cause inappropriate mitosis in cells lacking *rum1*⁺ (Moreno and Nurse 1994).

In summary, we have found that deletion of *rum1*⁺ greatly increases the severity of the *cdc18-K46* defect. This results in a rapid loss of cell viability and a failure to inhibit mitosis and cell division even though chromosomal DNA replication is incomplete. Together with our suppression experiments, these data indicate an important functional interaction between the *rum1*⁺ and *cdc18*⁺ genes and are consistent with the hypothesis that under these conditions the *rum1*⁺ gene product enhances *cdc18*⁺ gene function and restrains inappropriate cell division in vivo.

The Rum1 protein is a direct and potent inhibitor of Cdc2 kinase activity in vitro

This work and previous genetic studies have suggested a role for the *rum1*⁺ gene product in inhibiting mitosis and cell division (Fig. 2; Moreno and Nurse 1994). One potential target of this inhibition is the Cdc2 cyclin-dependent kinase, a critical regulator of both Start and mitosis in fission yeast (Nurse and Bissett 1981; Nurse 1990). Recently, a novel type of cell cycle regulator have been described that directly binds and inactivates CDKs. In budding yeast, for example, the *FAR1* and *SIC1* genes have been shown to encode inhibitors of the CDC28 CDK and block cell cycle progression in G₁ (Chang and Herskowitz 1991; Mendenhall 1993; Peter et al. 1993; Tyers and Futcher 1993; Donovan et al. 1994; Nugroho and Mendenhall 1994; Peter and Herskowitz 1994a; Schwob et al. 1994). In mammalian cells a growing number of CDK inhibitors, including the p21^{Cip1}, p27^{Kip1}, and p16^{Ink4a} inhibitory proteins, have been shown to regulate passage through the G₁/S transition (for review, see Sherr and Roberts 1995). Thus, it has been postulated that Rum1 functions as a direct inhibitor of the fission

yeast Cdc2 kinase (Moreno et al. 1994; Nurse 1994; Peter and Herskowitz 1994b).

To explore the possible biochemical functions of Rum1, we purified Rum1 as a glutathione S-transferase (GST) fusion protein from *Escherichia coli* and tested its effect on Cdc2-dependent phosphorylation in vitro. We found that GST-Rum1 specifically and potently inhibited histone H1 kinase activity in a dose-dependent manner, with >50% inhibition at 1 nM and 75% inhibition maximally (Fig. 3A).

The substantial residual activity observed at high concentrations of GST-Rum1 raised the possibility that Rum1 might inhibit some but not all forms of Cdc2 kinase. Therefore we separately immunoprecipitated either total Cdc2 kinase or that associated with Cdc13 (mitotic cyclin B) and evaluated the ability of GST-Rum1 to inhibit these kinases in vitro (Fig. 3B). GST-Rum1 specifically inhibited >95% of Cdc13-associated histone H1 kinase activity. Under the same conditions GST-Rum1 inhibited only ~80% of total Cdc2-associated activity. These results are consistent with the presence of Rum1-insensitive forms of Cdc2 kinase. Further biochemical studies with purified CDKs will be important in determining the relative sensitivity of specific Cdc2/cyclin complexes to Rum1.

Several lines of evidence support a specific inhibitory effect of Rum1 on Cdc2 kinase activity rather than non-specific kinase inhibition, contamination with phosphatases, or effects specific to the histone H1 substrate. First, GST-Rum1 did not inhibit histone H1 phosphorylation by the catalytic subunit of the cAMP-dependent protein kinase, even at concentrations several times higher than those used to inhibit Cdc2/cyclin B kinase (Fig. 3C). In addition, incubation of Cdc2-phosphorylated histone H1 with micromolar quantities of GST-Rum1 did not reduce histone H1 phosphorylation detectably (Fig. 3D). Finally, we purified the amino terminus of Cdc18 (which contains several copies of the Cdc2 consensus phosphorylation motif; Kelly et al. 1993) as a GST fusion protein. This GST-Cdc18 fusion protein was directly phosphorylated by Cdc2/cyclin B kinase in vitro; no phosphorylation was observed with the GST moiety alone as the substrate (Fig. 3E, lanes 1–4; data not shown). This phosphorylation was inhibited specifically and quantitatively in the presence of Rum1 (Fig. 3E, lane 5).

We sought to determine whether CDK inhibition might be mediated by physical interaction of Rum1 with either Cdc2 or Cdc13. *S. pombe* extracts were incubated in vitro with either GST or GST-Rum1, and associated proteins were retrieved with an affinity resin and washed extensively to remove nonspecifically bound components. Both Cdc2 and Cdc13 were detected in complexes with GST-Rum1 but not with GST alone (Fig. 4A,B). To address whether complex formation could be observed in vivo, we expressed a hemagglutinin (HA) epitope-tagged version of the Rum1 protein in wild-type *S. pombe* cells and immunoprecipitated the resulting extracts using antibodies to Cdc2 or Cdc13 (or a nonimmune antibody as a control). By immunoblotting with the anti-HA mono-

Figure 3. Rum1 is a direct and potent inhibitor of Cdc2 kinase activity in vitro. (A) Histone H1 kinase activity was measured as described (Moreno et al. 1989) in the presence of buffer alone (lane 1), 500 nM GST (lane 2), or 100 nM (lane 3), 10 nM (lane 4), 1 nM (lane 5), 0.1 nM (lane 6), or 0.01 nM GST-Rum1 (lane 7). Kinase activity is reported as a percentage of that observed when buffer was added. (B) *S. pombe* extracts were immunoprecipitated with antibodies to Cdc2 and Cdc13 (mitotic cyclin B) as described in Materials and methods. Histone H1 kinase activity was measured in the presence of either 500 nM GST (lanes 2,4) or 100 nM GST-Rum1 (lanes 1,3). Percent activity is given relative to GST controls. (C) The catalytic subunit of cyclic AMP-dependent protein kinase (PKA) was incubated with histone H1 in the presence of buffer alone (lane 1), 500 nM GST (lane 2), or 100 nM (lane 3) or 500 nM GST-Rum1 (lane 4) for 20 min at 30°C. (D) Histone H1 was phosphorylated as in A; kinase reactions were stopped by adding 25 mM EDTA (or in other experiments by boiling for 3 min and then cooling on ice). Samples were then incubated in the presence or absence of 3 μ M GST-Rum1 for an additional 20 min at 30°C and analyzed as above. (E) Fission yeast extracts were immunoprecipitated with antibodies to cyclin B (anti-Cdc13) or an irrelevant antibody (NI, nonimmune control). Immunoprecipitates were then assayed for the ability to phosphorylate GST (lane 2) or GST-Cdc18 substrates (lanes 1,3,4,5) in the presence of buffer (lanes 1–3), 500 nM GST (lane 4), or 50 nM GST-Rum1 (lane 5).

tibodies to Cdc2 or Cdc13 (or a nonimmune antibody as a control). By immunoblotting with the anti-HA monoclonal antibody 12CA5, a protein of ~27 kD (a size close to that predicted for the Rum1 protein based on conceptual translation) was detected that remained specifically associated with Cdc2 and Cdc13 (Fig. 4C). Taken together, our results demonstrate that Rum1 directly inhibits one or more forms of Cdc2 kinase, including the Cdc2/mitotic cyclin B kinase, and participates in specific protein complexes with Cdc2 and Cdc13 in vitro and in vivo.

Massive accumulation of Cdc18 protein links Cdc2 inhibition and re-replication

As mentioned previously, *rum1*⁺ was initially isolated as a gene that induces re-replication when strongly overexpressed in fission yeast (Moreno and Nurse 1994). Because we had observed that the *cdc18-K46* mutant phenotype was extremely sensitive to *rum1*⁺ gene dosage, we investigated whether overexpression of Rum1 affected the Cdc18 protein in vivo.

To test this possibility, a strain harboring a single chromosomal copy of the *cdc18*⁺ gene tagged with three

copies of the HA epitope (YMF187) was transformed with a plasmid expressing *rum1*⁺ under the control of the thiamine-repressible *nmt1*⁺ promoter (REP3X-*rum1*⁺) or with empty vector (REP3X) as a control. Transformants were selected and propagated in medium containing thiamine. At the start of the experiment, cells were washed extensively and incubated in medium lacking thiamine to induce expression of the *rum1*⁺ gene product.

We found that high levels of the Rum1 inhibitor in vivo triggered a dramatic increase in the Cdc18 protein, reaching a level ~25 $\times$ greater than normal (Fig. 5A). The bulk of this new Cdc18 protein was of increased mobility relative to Cdc18 from cells not expressing *rum1*⁺, perhaps suggesting an altered post-translational modification. The increase in Cdc18 protein was temporally correlated with re-replication of the genome, as the majority of cells reached DNA contents of $\geq 4C$ during this period (Fig. 5C). It should be noted that the overall ratio of DNA to protein remains relatively constant in cells overexpressing *rum1*⁺ (Moreno and Nurse 1994). Thus, Cdc18 protein is also greatly increased relative to total genome equivalents.

We investigated whether the accumulation of Cdc18

Figure 4. Physical interaction of Rum1 with Cdc2 and Cdc13 (mitotic cyclin B) proteins. Complex formation in vitro (A,B). *S. pombe* extracts were incubated with 500 ng of either GST (lane 2) or GST-Rum1 (lane 3). Complexes were recovered with glutathione-agarose and washed extensively with buffer. Associated proteins were separated by SDS-PAGE and immunoblotted with antibodies to Cdc2 (A) or Cdc13 (mitotic cyclin B) (B). An aliquot of the extract (10% input, lane 1) was analyzed on the same gel to allow comparison. (C) Complex formation in vivo. Extracts were prepared from a wild-type *S. pombe* strain expressing HA epitope-tagged Rum1 protein and immunoprecipitated with antibodies to Cdc2 (lane 2), Cdc13 (lanes 5, 7), or an irrelevant control antibody (nonimmune, lanes 3, 6). After extensive washing, immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with anti-HA monoclonal antibody 12CA5. One-tenth of the input extract (lanes 1, 4) was also analyzed for comparison.

strated less than twofold variation in *cdc18*⁺ mRNA levels during *rum1*⁺-induced re-replication (Fig. 5B). We therefore conclude that Rum1 promotes the accumulation of Cdc18 by a largely post-transcriptional mechanism. Given that Cdc18 is a highly labile protein (*t*_{1/2} 5 min; Muzi-Falconi et al. 1996), it is possible that Rum1 may act by reducing the rate of Cdc18 proteolysis.

To extend these observations, we examined whether inhibiting Cdc2 kinases by an independent route would also trigger an increase in Cdc18 protein and perhaps lead to re-replication. We exploited the fact that the budding yeast *SIC1* gene encodes an inhibitor of B-type cyclin-dependent kinases (Schwob et al. 1994) that is completely unrelated to *rum1*⁺. A plasmid expressing the *SIC1* gene under the control of the *nmt1*⁺ promoter (REP3X-*SIC1*) was transformed into strain YMF187 and induced as in the previous experiment. Fission yeast cells producing *SIC1* ceased division and became elongated with highly enlarged nuclei (Fig. 6A), much like cells overexpressing *rum1*⁺ (Moreno and Nurse 1994) or lacking the *cdc13*⁺ (mitotic cyclin B) gene (Hayles et al. 1994). Overexpression of *SIC1* caused Cdc18 to accumulate to high levels (Fig. 6B). Again, the major portion of this protein appeared to migrate more rapidly than Cdc18 from normal cells. We analyzed the DNA content of these cells by flow cytometry to determine whether they had also re-replicated. Consistent with the changes in cell morphology and Cdc18 protein, cells overexpressing *SIC1* initiated DNA replication in the absence of mitosis, yielding cells of predominantly 4C DNA content during this period (Fig. 6C). Thus, our data indicate that overexpression of either Rum1 or *SIC1* inhibitory proteins in *S. pombe* triggers a massive increase in Cdc18 protein levels that correlates with re-replication of the genome. We conclude that accumulation of Cdc18 protein is inhibited by CDKs and suggest that this may

be an important mechanism for establishing the temporal order of DNA replication and mitosis during the cell cycle.

Discussion

The findings presented in this report link a direct and potent inhibitor of CDKs to a key regulator of DNA replication and suggest a model for the proper alternation of S phase and mitosis in *S. pombe* (Fig. 7).

Previous work provided the genetic observation that overexpression of *rum1*⁺ leads to multiple rounds of DNA replication in the absence of mitosis (Moreno and Nurse 1994). The biochemical studies presented here demonstrate that the Rum1 protein is a potent inhibitor of the protein kinase activity of Cdc2/cyclin B complexes in vitro. We find that at nanomolar concentrations Rum1 quantitatively inhibits the phosphorylation of two distinct Cdc2 kinase substrates, histone H1 and a Cdc18 fusion protein. Moreover, we have shown that this inhibitory effect is mediated by the ability of Rum1 to form specific complexes with Cdc2 and Cdc13 (mitotic cyclin B) proteins in vitro and in vivo. Thus, it is likely that the re-replication phenotype observed when Rum1 is overproduced in fission yeast is a direct consequence of inhibition of Cdc2/cyclin B kinase activity. This explanation is supported by our observation that a structurally unrelated CDK inhibitor (encoded by the *SIC1* gene from budding yeast) also induces re-replication when overexpressed in *S. pombe*. It is also consistent with a number of other studies suggesting that CDKs can prevent the initiation of DNA replication. For example, addition of mitotic CDKs to *Xenopus* extracts prevents the formation of pre-replication complexes on sperm chromatin (Adachi and Laemmli 1994). Genetic studies of endoreduplication during *Drosophila* develop-

Figure 5. Overexpression of *rum1*⁺ triggers a massive increase in Cdc18 protein that correlates with re-replication. YMF187 transformants containing a thiamine-repressible *rum1*⁺ expression plasmid (REP3X-*rum1*⁺; lanes 2–6) or the empty vector as a control (REP3X; lane 1) were induced by washing and incubation in minimal medium lacking thiamine. Samples were taken immediately (lanes 1,2), or 12 hr (lane 3), 14 hr (lane 4), 16 hr (lane 5), or 18 hr (lane 6) after removal of thiamine. (A) Immunoblot analysis of Cdc18 and Cdc2 proteins. (*) A protein cross-reacting with the 12CA5 antibody is shown as an additional loading control. (B) Northern blot analysis of *cdc18* and *leu1* mRNAs. (C) Changes in DNA content during *rum1*⁺ overexpression were monitored by propidium iodide staining and flow cytometry.

ment clearly show the loss of mitotic cyclin expression in tissues undergoing re-replication of the fly genome (Lehner and O'Farrell 1990; Sauer et al. 1995). In fission yeast, loss of the Cdc2/cyclin B complex through deletion of *cdc13*⁺ (Hayles et al. 1994) triggers re-replication identical to that observed with overexpression of Rum1 and SIC1 (Moreno and Nurse 1994; this paper). In all of these cases it is likely that the mitotic CDK prevents the accumulation or activation of a factor or factors required for the initiation of DNA replication, but the identity of the relevant factors has remained elusive.

Our data indicate that inhibition of CDK strongly enhances the accumulation and biological function of Cdc18, a periodically expressed protein known to be in-

involved in triggering the onset of S phase in fission yeast. This connection between CDK activity and a regulator of DNA replication was discovered during the course of experiments aimed at identifying suppressors of a conditional *cdc18* mutant. We found that the *rum1*⁺ gene is capable of efficiently rescuing the lethality of this mutant when present on a multicopy plasmid or even as a single extra copy in the chromosome. Deletion of the *rum1*⁺ gene dramatically enhances the severity of the phenotype of the same *cdc18* mutant, causing rapid lethality and inappropriate entry into mitosis despite defects in DNA replication. The resulting *cut* phenotype is similar to that seen in cells deleted for the *cdc18*⁺ gene (Kelly et al. 1993). When the Rum1 protein (or the unrelated SIC1 protein) was overproduced from a strong *S. pombe* promoter, we observed a large increase in Cdc18 protein that was accompanied by re-replication of the genome. In light of the fact that Rum1 is a potent and direct inhibitor of Cdc2 kinase, the simplest interpretation of these data is that CDK activity normally acts to inhibit the accumulation of Cdc18 protein. It is possible that Cdc2 kinase affects the biochemical activity of Cdc18 protein as well as its accumulation, but at present we have no way to assess this point directly. Our interpretation clearly depends on the supposition that Rum1 affects Cdc18 accumulation solely through its inhibitory effect on CDKs. Whereas this seems likely, particularly in view of the fact that the unrelated CDK inhibitor SIC1 exerts similar effects when expressed in *S. pombe*, we cannot rule out the possibility that Rum1 and SIC1 share some common effector function in addition to their roles as CDK inhibitors.

Given the requirement for Cdc18 to initiate S phase (Kelly et al. 1993; Muzi-Falconi et al. 1996), it follows that inhibition of the accumulation and/or function of Cdc18 by mitotic CDK is sufficient to prevent re-replication. That inhibition of Cdc18 may also be necessary to prevent re-replication is indicated by the recent observation that massive overexpression of Cdc18 itself induces re-replication (Nishitani and Nurse 1995; Muzi-Falconi et al. 1996). Under these conditions the superabundance of Cdc18 presumably overcomes the inhibitory effects of cyclin-dependent kinase. Thus, the inhibition of Cdc18 by the mitotic Cdc2 kinase likely represents one of the mechanisms employed by the cell to ensure the alternation of S phase and mitosis. The accumulation of Cdc2/cyclin B kinase following initiation of DNA replication not only promotes mitosis but precludes reinitiation until after CDK activity is eliminated by cyclin B destruction at anaphase (for review, see King et al. 1994).

Our data indicate that the accumulation of Cdc18 protein when Rum1 is overexpressed is out of proportion to the relatively constant level of *cdc18*⁺ mRNA. This finding suggests that the accumulation is caused largely by effects on the rate of synthesis or degradation of Cdc18 protein. Cdc18 is normally a highly labile protein with a half-life of a few minutes (Muzi-Falconi et al. 1996). It is possible that inhibition of CDK leads to stabilization of the Cdc18 protein, although further work

Figure 6. Overexpression of the *SIC1* cyclin-dependent kinase inhibitor in fission yeast also triggers Cdc18 accumulation and re-replication. (A) Wild-type *S. pombe* containing a thiamine-repressible *SIC1* expression plasmid was grown in the presence (REP3X-*SIC1*, repressed) or absence of thiamine (REP3X-*SIC1*, induced). A strain containing the empty vector alone (REP3X) is shown for comparison. (B) YMF187 transformants containing REP3X (lane 1) or REP3X-*SIC1* (lanes 2–5) were induced as in the previous experiment and analyzed immediately (lanes 1,2), or 16 hr (lane 3), 20 hr (lane 4), or 22 hr (lane 5) after removal of thiamine. Immunoblot analysis of Cdc18 and Cdc2 proteins. (*) A protein cross-reacting with the 12CA5 antibody. (C) Flow cytometric analysis of DNA content during *SIC1* overexpression.

will be required to test this possibility. Given that Cdc18 is a phosphoprotein *in vivo* (M. Muzi-Falconi and T.J. Kelly, unpubl.) and can be a substrate for Cdc2/cyclin B *in vitro*, an attractive possibility is that direct phosphorylation of Cdc18 decreases its half-life. There are precedents for regulation of protein stability by phosphorylation. For example, the mammalian *V(D)J* recombinational activator protein RAG-2 appears to be targeted for proteolysis by phosphorylation at a Cdc2 consensus phosphorylation site (Lin and Desiderio 1993).

Genetic evidence indicates that the *cdc2⁺* gene is required for entry into S phase as well as mitosis (Nurse and Bissett 1981) and for *rum1⁺*-induced re-replication (Moreno and Nurse 1994). These and many other observations suggest that CDKs can have a role in activating S phase, as well as the inhibitory role emphasized here. These apparently paradoxical effects of CDKs can be reconciled if, for example, the identity of the cyclin partner determines whether a given CDK is an activator or inhibitor of S phase. Alternatively, it is possible to imagine models in which low levels of kinase activity promote S phase, whereas higher levels inhibit re-replication and promote mitosis. Such models provide a particularly simple solution to the problem of ensuring the alternation of S phase and mitosis. The potential for CDKs to have either a positive or a negative effect on S-phase entry may explain some aspects of the function of the CDK inhibitor Rum1. Overexpression of Rum1 blocks mitosis and allows re-replication; under these conditions there is presumably enough (or the right kind of) CDK activity to promote S phase but not to allow mitosis. On the other hand, the effect of deletion of the *rum1⁺* gene is to accelerate the onset of S phase under conditions

Figure 7. Functional interactions among the *rum1⁺*, *cdc2⁺*, and *cdc18⁺* gene products define a pathway that regulates the alternation of DNA replication and mitosis. The Rum1 protein is a potent and direct inhibitor of the Cdc2/mitotic cyclin B (Cdc13) kinase complex. Overexpression of Rum1 in fission yeast cells constitutively inhibits the mitotic CDK, leading to a state permissive for DNA replication in the absence of mitosis. This state can also be reached by overexpression of the unrelated *SIC1* protein (a CDK inhibitor from *S. cerevisiae*) or by preventing synthesis of the cyclin B component of the kinase (by deletion of the *cdc13⁺* gene). The absence of active Cdc2/cyclin B kinase eliminates a critical restraint against re-replication by allowing the high-level accumulation of Cdc18 protein, which is a major rate-limiting regulator of S-phase entry. The inhibition of Cdc18 expression by mitotic CDKs can explain the proper alternation of DNA replication with mitosis, as ectopic expression of Cdc18 to high levels is sufficient to induce re-replication of the genome.

that normally lead to delay of DNA replication, such as nitrogen starvation and small cell size (Moreno and Nurse 1994). Under these circumstances, removing the inhibitory effect of Rum1 may augment the S phase-promoting activity of the appropriate CDK.

The budding yeast *CDC6* gene encodes a protein with significant amino acid sequence homology to Cdc18 and may be regulated in a similar manner. As in the case of Cdc18, expression of *CDC6* is required in each cell cycle for the initiation of DNA replication. Recent work suggests that the *CDC6* protein may interact with the origin recognition complex (Liang et al. 1995). In budding yeast, like fission yeast, high levels of mitotic CDKs inhibit entry into S phase. The inhibitory effect is caused in part by transcriptional repression of the *CLN* genes, which encode G_1 cyclins required for the transition through Start (Amon et al. 1993). It is also possible that CDKs may inhibit the accumulation and/or activation of *CDC6* after the beginning of S phase, but further work will be required to examine this hypothesis. Intriguingly, expression of B-type cyclins in *Saccharomyces cerevisiae* appears shortly after the initiation of DNA synthesis and persists until activation of the cyclin destruction machinery in anaphase and pre-Start G_1 (Amon et al. 1994; Irmiger et al. 1995), whereas periodic accumulation of *CDC6* protein is restricted to late anaphase and early G_1 intervals (Piatti et al. 1995). We suspect that the inhibition of Cdc18/*CDC6* family members by CDKs may be a general mechanism for ensuring the alternation of mitosis and S phase.

Materials and methods

Growth and manipulation of fission yeast strains

Strains used in this study are listed in Table 1 and are derived from strains described previously (Kelly et al. 1993; Muzi-Falconi et al. 1996). *S. pombe* was grown in rich (YE5S) or minimal medium (EMM) containing the required supplements. Genetic crosses and tetrad analysis were performed using standard methods (Moreno et al. 1991), with the necessary modifications to suppress the sterility of the *rum1*⁺ deletion (Moreno and Nurse 1994).

Induction of *nmt1* promoter constructs and analysis of gene expression

Strains transformed with plasmids containing the thiamine-repressible *nmt1* promoter (REP3X and its derivatives) were isolated and maintained in minimal medium containing 5 μ g/ml of thiamine. Cells were washed five times and incubated in medium lacking thiamine to induce expression, which begins ~12–14 hr later (Maundrell 1990). Samples were taken for RNA

and protein preparation, and for determination of DNA content by propidium iodide staining and flow cytometry as described previously (Kelly et al. 1993). RNA samples were prepared by glass bead lysis and purification using the RNeasy system (Qiagen). Electrophoresis, blotting, hybridization, washing, and autoradiography were performed using GeneScreen Plus (NEN, Dupont) according to the manufacturer's directions. Quantitation of ³²P signals was performed using a Molecular Dynamics PhosphorImager. Total *S. pombe* protein was prepared by glass bead lysis in SDS sample buffer and boiling. Proteins were separated by 12% SDS-PAGE, transferred to nitrocellulose, and probed with C2 (anti-Cdc2), 12CA5 (anti-hemagglutinin), and SP4 (anti-Cdc13) primary antibodies and the appropriate secondary antibody-horseradish peroxidase conjugates. Immunoblots were developed using ECL (Amersham). Quantitation was performed by serial dilution of samples and densitometric analysis of appropriate exposures using the NIH Image software package.

Cloning of a *cdc18*-K46 suppressor

A library was constructed in pON163 (Weilguny et al. 1991) from partially *Sau*3A-digested and size-fractionated *S. pombe* genomic DNA (P.V. Jallepalli and T.J. Kelly, unpubl.). The genomic library was introduced into PJ18 by electroporation (Kelly et al. 1993). Transformants were obtained on minimal medium lacking uracil at 25°C and then replica-plated to the same medium at 36°C, or selected directly at 36°C after a 24-hr recovery period at 25°C. In each case, suppression of temperature sensitivity was linked to the *ura4*⁺ marker on the plasmid. Plasmids were then recovered by transformation of total *S. pombe* DNA into *E. coli*.

Restriction analysis and Southern hybridization established that five plasmids with overlapping inserts had been recovered that were unrelated to *cdc18*⁺. A common 6.1-kb *Sal*-*Kpn* fragment contained the rescuing activity upon retransformation into PJ18. A minimum suppressing fragment of ~2.5 kb was defined by subcloning and unidirectional deletion analysis (Erase-A-Base, Promega) and sequenced manually (Sequenase) or semiautomatically (Prism, ABI).

Purification of GST fusion proteins

The entire *rum1*⁺ open reading frame (ORF) (nucleotides 129–821) and a portion of the *cdc18*⁺ ORF corresponding to the amino terminus (nucleotides 13–227) were amplified through PCR and subcloned as *Bam*HI fragments into the pGEX-3X bacterial expression vector (Pharmacia). *E. coli* strain BL21 was transformed with the resulting plasmids, cultured in Luria broth plus 50 mg/liter of carbenicillin at 37°C, and induced for 3 hr with 0.4 mM IPTG. Subsequent purification of GST, GST-Rum1, and GST-Cdc18 fusion proteins on glutathione-agarose was performed according to Connell-Crowley et al. (1993).

Table 1. Fission yeast strains used in this study

TK8	<i>h</i> ⁺ <i>leu1-32 ade6 ura4-D18</i>
PJ18	<i>h</i> ⁺ <i>cdc18-K46 leu1-32 ade6 ura4-D18</i>
PJ101	<i>h</i> ⁺ $\Delta rum1::ura4^+$ <i>leu1-32 ade6 ura4-D18</i>
PJ109	<i>h</i> ⁺ <i>cdc18-K46 $\Delta rum1::ura4^+$ leu1-32 ade6 ura4-D18</i>
YMF15	<i>h</i> ⁺ $\Delta cdc18:: (nmt1-cdc18^+ leu1^+)$ <i>leu1-32 ura4-D18</i>
YMF187	<i>h</i> ⁺ <i>3HA-cdc18⁺ leu1-32 ura4-D18</i>

Protein kinase assays and protein-protein interaction studies

Native *S. pombe* extracts were prepared by glass bead lysis in the presence of HB buffer (Moreno et al. 1991). Cdc2-dependent phosphorylation of histone H1 was measured using the assay described previously (Moreno et al. 1989), with buffer or purified proteins added as indicated. For immunoprecipitation kinase assays, 200 μ g of extract was incubated with C2 (anti-Cdc2) or min56 (anti-Cdc13) antibodies or an irrelevant antibody for 1 hr, and immune complexes were collected with 30 μ l of protein A-Sepharose. Immunoprecipitates were washed six times with HB buffer and incubated in 50 μ l of kinase buffer (HB buffer supplemented with 200 μ M ATP [containing 40- μ Ci/ml [γ - 32 P]ATP and 1 mg/ml of the indicated substrate] with or without added proteins for 20 min at 30°C. Phosphorylation was analyzed by 12% SDS-PAGE and quantitated using a PhosphorImager.

In vitro binding experiments were performed by incubating 150 μ g of extract with 500 ng of GST or GST-Rum1 (in a final volume of 1 ml, made up with HB buffer) at 0°C for 90 min. Complexes were retrieved with 30 μ l of glutathione-agarose and washed six times with 1 ml of HB buffer. Associated proteins were separated by 12% SDS-PAGE and immunoblotted for Cdc2 and Cdc13 as described above.

To examine in vivo complex formation, the *rum1*⁺ ORF was tagged at the carboxyl terminus with three copies of the HA epitope and placed under the control of the *nmt1*⁺ promoter (pREP4X-*rum1*-HA3). Wild-type fission yeast cells harboring this plasmid were grown for 15 hr in medium lacking thiamine to induce expression, and soluble native extracts were prepared as described above. Protein extract (400 μ g) was incubated with antibodies to Cdc2 (C2) or Cdc13 (min56 or edi56) or a nonimmune control antibody for 1 hr, and immune complexes were collected with 30 μ l of protein A-Sepharose. Immunoprecipitates were washed six times with HB buffer, boiled in SDS sample buffer, and analyzed by 12% SDS-PAGE. Rum1 protein was detected by immunoblotting with the 12CA5 anti-HA monoclonal antibody.

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