The Cdk inhibitors p25\textsuperscript{rum1} and p40\textsuperscript{SIC1} are functional homologues that play similar roles in the regulation of the cell cycle in fission and budding yeast

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Accepted 7 January 1998; published on WWW 23 February 1998

INTRODUCTION

Cell cycle transitions are regulated by the activation and inactivation of cyclin-dependent kinases (Cdks). Cdks are regulated by association with positive regulatory subunits known as cyclins, negative regulators known as Cdk inhibitors and by phosphorylation (reviewed by Martín-Castellanos and Moreno, 1997). In yeast a single Cdk, encoded by CDC28 in \textit{S. cerevisiae} or cdc\textsuperscript{2} in \textit{S. pombe}, regulates cell cycle progression in association with different cyclins (Nurse and Bissett, 1981; Piggott et al., 1982; Reed and Wittenberg, 1990). Budding yeast \textit{G}_1 cyclins Cln1, Cln2 and Cln3 drive cells through \textit{G}_1 by activating the Cdc28 protein kinase; Clb5 and Clb6, promote \textit{S} phase and Clb1 through Clb4 entry into mitosis (reviewed by Nasmyth, 1996). In fission yeast, cig\textsuperscript{2} is the cyclin that regulates entry into \textit{S}-phase, while cdc13 is the mitotic cyclin (reviewed by Stern and Nurse, 1996). In both yeasts, mitotic cyclins can perform the function of \textit{S}-phase cyclins which means that there is some degree of functional redundancy between Clb1-6 in budding yeast and between cig\textsuperscript{2} and cdc13 in fission yeast (Fisher and Nurse, 1996; Monderset et al., 1996; Schwob et al., 1994). Cdk/cyclin activity in \textit{S}-phase and \textit{G}_2 is also necessary to prevent another round of DNA replication within the same cell cycle (Hayles et al., 1994; Dahmann et al., 1995).

Cdk activity is negatively regulated by binding of Cdk inhibitors (CKI). These are proteins that induce cell cycle arrest or delay in response to intracellular or extracellular signals. In mammalian cells, addition of transforming growth factor \(\beta\) (TGF\(\beta\)) arrests the cultures in late \textit{G}_1 (Laiho et al., 1990). A similar situation occurs in budding yeast after addition of sexual pheromones (Herskowitz, 1995). Some CKIs function in response to extracellular signals, but others appear to be part of the intrinsic cell cycle machinery. CKIs are able to associate with the Cdk subunit, the cyclin or the Cdk/cyclin complex to inhibit its kinase activity. In animal cells, two structurally defined classes of Cdk inhibitors have been described so far (Sherr and Roberts, 1995; Elledge et al., 1996; Martín-Castellanos and Moreno, 1997). Members of the Ink4 family (p15, p16, p18 and p19) specifically bind to Cdk4 and Cdk6, acting as inhibitors of cyclin Ds. The Kip/Cip family members (p27, p57 and p21), however, seem to function only in heterotrimeric complexes with the Cdk and the cyclin. They bind to and inhibit Cdk2, Cdk3, Cdk4 and Cdk6 kinases but are less effective towards Cdc2/cyclinB, suggesting that p21 and p27 are not universal inhibitors of Cdks and display selectivity for \textit{G}_1/S Cdk/cyclin complexes (Harper et al., 1995).

In budding yeast, two Cdk inhibitors have been described, Far1 and p40\textsuperscript{SIC1}. Far1 binds to and inhibits Cdc28/Cln complexes to mediate pheromone cell cycle blockage (Chang and Herskowitz, 1990; Peter et al., 1993; Peter and Herskowitz, 1994). In contrast to Far1, p40\textsuperscript{SIC1} functions as an intrinsic...
component of the cell cycle machine in that its protein levels or activity is not dependent on external factors. p40SIC1 was first described as a tightly bound substrate of Cdc28 (Mendenhall et al., 1987; Nugroho and Mendenhall, 1994; Reed et al., 1985). p40SIC1 accumulates as cells exit from mitosis and disappears just before the initiation of DNA replication (Donovan et al., 1994; Schwob et al., 1994). Destruction of p40SIC1 is dependent on the activity of the ubiquitin conjugating enzyme, Cdc34 (Goebel et al., 1988; Schwob et al., 1994). It has been shown that removal of p40SIC1 results in premature entry into S-phase in a high percentage of unbudded cells. In fact, DNA replication and budding occur at different times in sic1 null mutants (Schneider et al., 1996). p40SIC1 inactivation is essential for S phase initiation since it exerts its inhibitory activity on the Clb5-Cdc28 and Clb6-Cdc28 complexes (Schwob et al., 1994). It has been proposed that p40SIC1 couples DNA replication to the completion of Start (Moreno and Nurse, 1994; Schneider et al., 1996). Recently it has been shown that p40SIC1 couples DNA replication to the completion of Start (Moreno and Nurse, 1994; Labib and Moreno, 1996). Lack of p40SIC1 inactivation is essential for S phase initiation since it exerts its inhibitory activity on the Clb5-Cdc28 and Clb6-Cdc28 complexes (Schwob et al., 1994). It has been proposed that p40SIC1 couples DNA replication to the completion of Start (Moreno and Nurse, 1994; Tyers, 1996).

In fission yeast, p25rum1 plays a central role in the regulation of the G1 phase (Moreno and Nurse, 1994; reviewed by Labib and Moreno, 1996). Lack of rum1+ in small cells is lethal because cells undergo premature S-phase immediately after mitosis (Moreno and Nurse, 1994). p25rum1 is a potent inhibitor of the cdc2/cdc13 mitotic kinase (Correa-Bordes and Nurse, 1995; Jallepalli and Kelly, 1996; Martin-Castelanos et al., 1996). Recently it has been shown that p25rum1 is also required to promote cdc13 proteolysis in G1 cells (Correa-Bordes et al., 1997). Similarly to p40SIC1 in S. cerevisiae, p25rum1 also oscillates during the cell cycle and is present from anaphase until the end of G1 where it is destroyed through the ubiquitin-dependent proteasome pathway (Benito et al., 1998).

In this paper we show that the Cdk inhibitors p25rum1 and p40SIC1 are functional and structural homologues that may perform a similar function in the regulation of the cell cycle in fission yeast and budding yeast.

MATERIALS AND METHODS

Yeast strains and methods

The fission yeast strains used in this study were (Sp1) h- 972, (Sp20) h+ ade6-704 leu1-32 ura4-d18, (Sp282) h+ rum1::ura4* ura4-d18 leu1-32 ade6-M216, (Sp544) h-/+ rum1::ura4*/rum1::ura4* ura4-d18 ura4-d18 leu1-32 ade6-M216/ad6-M216, (Sp177) h+ cdc10-129, (Sp224) h+ cdc10-129 rum1::ura4* ura4-d18 leu1-32, (Sp63) h+ ade6-704 leu1-32 ura4-d18 intest-nmt-rum1* (sup3.5), (Sp507) h+ ade6-704 leu1-32 ura4-d18 intest-nmt-sic1-SUO-3 (sup3.5), (Sp563) h+ rum1::ura4*/ura4* ura4-d18 leu1-32 intest-nmt-rum1* (sup3.5), (Sp564) h+ rum1::ura4*/ura4* ura4-d18 intest-nmt-rum1* (sup3.5), (Sp565) h+ rum1::ura4*/ura4* ura4-d18 intest-nmt-rum1* (sup3.5). The budding yeast strains were all generated from W303 (Sc20, a ade2 trpl can1 leu2 his3 Δura3), 15 Dau (Sc55, α ade2 leu2 his3 trpl Δura3), 15 Dau (Sc55, α cdc4-1 leu2 his3 Δura3) and 15 Dau cdc4-1 (Sc55, α cdc4-1 leu2 his3 Δura3). Growth conditions and strain manipulations for fission and budding yeast were as previously described (Moreno et al., 1991; Sherman, 1991). Yeast transformation was carried out using the lithium acetate transformation protocol (Moreno et al., 1991) and integrants were confirmed by Southern blotting. All experiments in liquid culture with fission yeast were carried out in minimal medium (EMM) containing the required supplements, starting with a cell density of 2-4×10^6 cells/ml, corresponding to mid-exponential phase growth. To induce expression from the nmt1 promoter, cells were grown to mid-exponential phase in minimal medium (EMM) containing 5 μg/ml thiamine, then spun down and washed four times with minimal medium, and resuspended in minimal medium lacking thiamine at a density calculated to produce 4×10^6 cells/ml at the time of peak expression from the nmt1 promoter. Overexpression of rum1+ deletion fragments in S. pombe was carried out transfecting the different rum1 truncates alleles from pAS2 to pREP1 (Durfee et al., 1993; Maundrell, 1989, 1993) after digesting with NdeI-BamHI. For galactose induction, budding yeast cells were grown in YEP supplemented with 2% raffinose (YEPR) at 28°C until the culture reached mid-exponential phase. Then galactose was added to a final concentration of 2.5%.

Plasmids

The plasmids used in this work are the following: pREP3X-rum1+ contains the 1.5 kb cDNA cloned in pREP3X as a SalI-BamHI fragment (Moreno and Nurse, 1994). pREP3X-SIC1 contains a 1.6 kb BamHI-BglII from pb148 (a gift of M. Mendenhall, University of Kentucky) cloned at the BamHI site of pREP3X. pREP3X-FAR1 contains a 3.2 kb Xhol-NotI from pRS129 (a gift of M. Piter, University of California, San Francisco) cloned in pREP3X. pREP1-KIP1 contains a 0.6 kb Nhel-BamHI fragment from pMEP4 (a gift from Kornelia Poljak and Joan Massagué, Memorial Sloan-Kettering Cancer Center) cloned pREP1 digested with Xbal-BamHI. pNURI-IN7A contains a 1 kb fragment from pBS-p16 (a gift of Manuel Serrano and David Beach, Cold Spring Harbor Laboratory) cloned in SpeI-XhoI in pNURI. pRT2-rum1+ contains a 3.8 kb PstI-BamHI rum1+ genomic fragment cloned in pRT2. S. cerevisiae plasmid YipG3-SIC1 contains a 1.6 kb BamHI-BglII from pb148 (a gift of M. Mendenhall, University of Kentucky) cloned at the BamHI site of YipG3. YipG3-rum1+ contains a 0.8 kb BamHI fragment amplified by PCR with the oligonucleotides 5′-GGTTTTTTGGATCCCTGTTAGTGTTGAGAGTCCCG-3′ and 5′-GGCCATTTATATACAAGAGCAAGGATCCACAC-3′ cloned at the BamHI site in YipG3.

Flow cytometry and microscopy

About 10^7 cells were spun down, washed once with water, fixed in 70% ethanol and processed for flow cytometry or DAPI staining as described previously (Moreno et al., 1991; Sazer and Sherwood, 1990). A Becton-Dickinson FACScan was used for flow cytometry. For microscopy, cells were photographed using a phase contrast Zeiss axiophot photomicroscope.

Protein-protein interactions using the two hybrid system

Protein interactions were analysed using the two hybrid system (Durfee et al., 1993). rum1+ fragments were amplified by PCR, cloned into the NcoI-BamHI sites of pACT2 and sequenced. PCR amplifications were performed using different oligonucleotides including the NcoI and BamHI restriction sites. For rum1 (1-147) we used the primers S1 (5′-ATATACCATGGAACCTCCTACACAC-ACC′-3′ and SD (5′-TATATGCATCTTATGCTGACAAACCAGATTGGTG- G′-3′); for rum1 (52-147), S6 (5′-ATATACCATGGAACCTCCTACACAC- ACC′-3′) and SD; and for rum1 (67-147), SC (5′- ATATACCATGGAACCTCCTACACAC-ACC′-3′) and SD; for rum1 (84-147), SA (5′-ATATACCATGGAACCTCCTACACAC-ACC′-3′) and SD. A 924 bp fragment coding for the cdc13 307 carboxy-terminal amino acids was amplified by PCR using the primers S18 (5′-ATATACCATGGAACCTCCTACACAC-ACC′-3′) and S20 (5′-TATATGCATCTTATGCTGACAAACCAGATTGGTG- G′-3′). The S. cerevisiae Y190 strain was transformed and β-galactosidase activity was analysed in the transformants as described (Guanante, 1983).
RESULTS

Complementation of rum1 deletion by Cdk inhibitors

The first goal of these studies was to establish if Cdk inhibitors from other organisms were able to rescue the phenotypes of fission yeast cells deleted for the rum1\(^+\) gene. Deletion of the rum1\(^+\) in the temperature sensitive cdc10-129 mutant background reduces the maximal temperature at which the cdc10-129 mutant can grow from 32\(^\circ\)C to 28\(^\circ\)C (Fig. 1A), since at higher temperatures cells enter mitosis inappropriately without having replicated their chromosomes. This defect can be rescued by expressing the rum1\(^+\) gene from its own promoter (Fig. 1B; pIRT2-rum1\(^+\)) or from the thiamine repressible nmt1 promoter in minimal medium containing 5 \(\mu\)g/ml of thiamine (Fig. 1B; pREP3X-rum1\(^+\)). This promoter allows moderate levels of gene expression in the presence of thiamine and high levels of gene expression in the absence of thiamine (Maundrell, 1989, 1993). To test whether other Cdk inhibitors from budding yeast or animal cells were able to rescue the rum1\(^+\) phenotype, we subcloned the cDNAs of FAR1, SIC1, CIP1 and KIP1 in pREP3X and transformed each of these constructions into the cdc10-129 rum1\(^+\) strain. We found that expression of SIC1 in the presence of thiamine was sufficient to restore growth of the double mutant at 32\(^\circ\)C (Fig. 1B). The rest of the Cdk inhibitors did not rescue this phenotype even when produced at high level in minimal medium without thiamine.

In fission yeast there is functional redundancy between cig2 and cdc13 cyclins in G\(_1\) and inhibition of both cdc2/cig2 and cdc2/cdc13 kinase activities is required for G\(_1\) arrest (Stern and Nurse, 1997). Cells lacking rum1\(^+\) do not arrest in G\(_1\) in response to nitrogen starvation and to mating pheromones (Moreno and Nurse, 1994). Therefore, rum1\(^+\) cells are sterile and unable to undergo meiosis (Fig. 2B). The rum1\(^+\) mating and meiotic deficiencies were completely suppressed when a rum1\(^+\) cDNA was expressed using the nmt1 promoter under repressing conditions (Fig. 2C). We used the constructions described above containing the S. cerevisiae and animal cells Cdk inhibitors cDNAs to test if these genes were able to rescue the conjugation and meiotic defects of cells deleted for the rum1\(^+\) gene. Expression of the SIC1 gene was able to complement the mating defect of the h\(^-\)rum1\(^+\) strain (Fig. 2D, left) and the meiotic defect of a h\(^+/\)h\(^-\) rum1\(^+\)/rum1\(^-\) homzygous diploid (Fig. 2D, right). SIC1 also partially rescued the inability of rum1\(^+\) deleted cells to arrest in G\(_1\) in response to nitrogen starvation (Fig. 3). In summary, these experiments show that the S. cerevisiae SIC1 gene complements all the phenotypes of the rum1\(^+\) deletion strain and that none of the other Cdk inhibitors analysed (Far1, p21\(^{CIP1}\) and p27\(^{KIP1}\)) rescued any of these defects. We have also tried the p16\(^{INK4a}\) Cdk inhibitor and did not find complementation of any of the phenotypes of the rum1\(^-\) described above (data not shown).

Overproduction of SIC1 in fission yeast generates a phenotype very similar to the overproduction of rum1\(^+\)

In fission yeast, the cdc2/cdc13 complex plays a dual role during the cell cycle. First, it promotes entry into mitosis and, second, it inhibits re-initiation of extra rounds of DNA replication within a single cell cycle (Fisher and Nurse, 1996; Hayles et al., 1994). Overproduction of rum1\(^+\) in G\(_2\) inhibits the protein kinase activity associated with cdc2/cdc13 blocking entry into mitosis, allowing multiple rounds of DNA replication leading to an increase in DNA content (Moreno and Nurse, 1994; Fig. 4A). Constitutive low level expression of rum1\(^+\) from the nmt1 promoter in minimal medium containing thiamine induces some degree of diploidization (about 2-5% of the cells are diploids). These diploids can be detected by plating the cells in medium containing phloxin B, where haploid colonies are pink while diploid colonies are red (Moreno et al., 1991). Low level expression of SIC1 in S. cerevisiae SIC1 gene rescues the growth defect at 32\(^\circ\)C of the cdc10-129 rum1\(^+\) mutant. (A) Deletion of the rum1\(^+\) gene reduces the maximal temperature at which the cdc10-129 mutant can grow from 32\(^\circ\)C to 28\(^\circ\)C. Wild-type 972 h\(^+\) (Sp1), cdc10-129 (Sp177) and cdc10-129 rum1\(^+\) (Sp224) cells were grown at 25\(^\circ\)C in YES medium until early exponential phase and then plated out in YES agar and incubated for 3 days at 25\(^\circ\)C, 28\(^\circ\)C, 30\(^\circ\)C, 32\(^\circ\)C and 37\(^\circ\)C. (B) The cdc10-129 rum1\(^+\) lea1-32 strain (Sp224) was transformed with pREP3X, pREP3X-rum1\(^+\), pIRT2-rum1\(^+\), pREP3X-SIC1, pREP3X-FAR1, pREP3X-CIP1 and pREP1-KIP1. Transformants were allowed to grow at 25\(^\circ\)C in minimal medium containing 5 \(\mu\)g/ml thiamine and then streaked out onto the same medium and incubated at 25\(^\circ\)C and 32\(^\circ\)C for 3 days.

![Fig. 1. Expression of the S. cerevisiae SIC1 gene rescues the growth defect at 32\(^\circ\)C of the cdc10-129 rum1\(^+\) mutant. (A) Deletion of the rum1\(^+\) gene reduces the maximal temperature at which the cdc10-129 mutant can grow from 32\(^\circ\)C to 28\(^\circ\)C. Wild-type 972 h\(^+\) (Sp1), cdc10-129 (Sp177) and cdc10-129 rum1\(^+\) (Sp224) cells were grown at 25\(^\circ\)C in YES medium until early exponential phase and then plated out in YES agar and incubated for 3 days at 25\(^\circ\)C, 28\(^\circ\)C, 30\(^\circ\)C, 32\(^\circ\)C and 37\(^\circ\)C. (B) The cdc10-129 rum1\(^+\) lea1-32 strain (Sp224) was transformed with pREP3X, pREP3X-rum1\(^+\), pIRT2-rum1\(^+\), pREP3X-SIC1, pREP3X-FAR1, pREP3X-CIP1 and pREP1-KIP1. Transformants were allowed to grow at 25\(^\circ\)C in minimal medium containing 5 \(\mu\)g/ml thiamine and then streaked out onto the same medium and incubated at 25\(^\circ\)C and 32\(^\circ\)C for 3 days.](Image)
pombe also causes a similar degree of diploidization and high level expression of SIC1 induces re-replication (Fig. 4B; see also Jallepalli and Kelly, 1996). The cells started to elongate after 18 hours of induction and showed a more prominent nucleus after DAPI staining (Fig. 5D). The re-replication phenotype induced by p40SIC1 is delayed by about 4 hours compared to the one induced by p25 rum1 (Fig. 4B). After prolonged incubation, these cells presented a nucleus of a similar size to cells overexpressing rum1 + (compare Fig. 5B and D). We have also induced either rum1 + or SIC1 for 14 hours at 32°C and then plated cells on medium containing thiamine and incubated at 32°C. Crosses were set for the haploid strains and cells were photographed after 3 days using a Zeiss axiophot phase contrast microscope. (A) Wild-type controls. (B) Mutant cells transformed with pREP3X. (C) Mutant cells transformed with pREP3X-rum1 +. (D) Mutant cells transformed with pREP3X-SIC1.

Fig. 2. S. cerevisiae SIC1 gene rescues the mating and meiotic defect of fission yeast cells deleted for rum1 +. Haploid h- rum1Δ leu1-32 (Sp282) and diploid h+/h+ rum1Δ/rum1Δ leu1-32/leu1-32 (Sp544) cells were transformed with pREP3X, pREP3X-rum1 + and pREP3X-SIC1. Cells were plated out in minimal medium containing 5 μg/ml thiamine and incubated at 32°C. Crosses were set for the haploid strains and cells were photographed after 3 days using a Zeiss axiophot phase contrast microscope. (A) Wild-type controls. (B) Mutant cells transformed with pREP3X. (C) Mutant cells transformed with pREP3X-rum1 +. (D) Mutant cells transformed with pREP3X-SIC1.

Fig. 3. Expression of the S. cerevisiae SIC1 gene rescues the inability of rum1Δ mutants to arrest in G1 in response to nitrogen starvation. Cells deleted for the rum1 gene were transformed with the plasmids pJK148-81X, pJK148-3X-rum1 + and pJK148-3X-SIC1 and integrants were isolated. Wild-type control 972 h- (Sp1) and the different integrants in the rum1Δ background were grown at 25°C in minimal medium containing 5 μg/ml thiamine until early exponential phase and then shifted to minimal medium lacking nitrogen. Samples for FACS analysis were taken at the indicated times.

Overproduction of rum1 + in S. cerevisiae causes a phenotype similar to the inactivation of all the Clb cyclins

The experiments described above indicate that p40SIC1 when expressed in fission yeast can carry out the functions of p25rum1. That is, it can rescue the sterility and meiotic defects of the rum1Δ and the synthetic lethality of the cdc10-129 rum1Δ strain at 32°C. In addition, overproduction of SIC1 in S. pombe produces phenotypes similar to the overproduction of rum1 +.

Next, we decided to study if p25rum1 behaves as a cell cycle inhibitor when expressed in S. cerevisiae. For this purpose, we expressed rum1 + or SIC1 from the GAL1 promoter in the S. cerevisiae strain W303. Integrants were selected in minimal medium containing glucose and confirmed by Southern blot analysis. Induction of the SIC1 gene in
galactose causes a cell cycle delay both in G₁ and G₂ with cells containing elongated multi-budded cells (Figs 6A, 7B; see also Nugroho and Mendenhall, 1994). Cells overexpressing rum1⁺ arrested both with unbudded and large budded cells (Fig. 7D). Some of the large budded cells have multiple buds (Fig. 7D). This phenotype is reminiscent of the lack of all the Clb cyclins or the cdc4, cdc34, cdc53 or skp1 mutants (Bai et al., 1996; Mathias et al., 1996; Schwob et al., 1994; Yochem and Byers, 1987). Although the bud arrest morphology is not homogeneous, these cells are homogeneously arrested in G₁ with 1C DNA content (Fig. 6B). This result suggests that high level expression of p25rum1 in budding yeast generates a phenotype consistent with a preferential inhibition of Cdc28/Clb kinase complexes in vivo.

S. cerevisiae cdc4-1 mutants lacking SIC1 do not arrest in G₁ when shifted to the restrictive temperature (Schwob et al., 1994). We found that expression of rum1⁺ in this double mutant background was able to restore the G₁ arrest of cdc4-1 mutants at 37°C (Fig. 8), suggesting that at least in this situation rum1 is able functionally replace Sic1 in vivo, inhibiting Cdc28/Clb activity and preventing entry into S phase.

The Cdk inhibitory domain of p25rum1 is conserved in p40SIC1

We have mapped the cyclin interacting domain and the Cdk inhibitory domain in p25rum1 to a region of 80 amino acids in the middle of the protein, from amino acid 67 to 147. To map the cyclin binding domain in p25rum1 we used the two hybrid interaction system. p25rum1 strongly interacts with p56cdc13 cyclin. Indeed, expression of either rum1⁺ or cdc13⁺ individually in S. cerevisiae from the ADH promoter is lethal but cells co-expressing rum1⁺ (or certain deletions of rum1⁺) and cdc13⁺ are alive. Using different deletions of the rum1⁺ gene we have mapped the interaction domain between p25rum1 and p56cdc13 to a central region in p25rum1 extending from amino acids 67 to 147 (Fig. 9B). For the mapping of the Cdk
inhibitory domain, we overexpressed the same deletions of the
rum1+ gene in S. pombe cells from the nmt1 promoter. In this
case, we found that expression of a truncated version of
p25rum1 from amino acid 67 to 147 was sufficient to block the
cell cycle (Fig. 9A). A similar result was obtained when we
expressed the mutant 52 to 147 (Fig. 9A). Overexpression of
the 84 to 147 mutant did not show any phenotype (Fig. 9A).
We could detect more elongated cells and diploids when we
expressed the 52 to 147 or the 67 to 147 truncations than with
wild-type rum1+ in medium containing thiamine (Fig. 9A),
suggesting that these truncated proteins are either more active
as inhibitors or more stable than full length p25rum1.

p25 rum1 and p40 SIC1 seem to perform similar functions as
cell cycle regulators in fission and budding yeast. But are they
true homologues? Protein sequence alignment between
p25rum1 and p40 SIC1 revealed only a 23% identity over the
whole protein (Fig. 10). However, this homology rises to 33%
within the 80 amino acids domain we have defined in p25 rum1
to interact with cdc13 and to inhibit the cell cycle in vivo.
Interestingly, this domain corresponds to the C-terminal region
of p40 SIC1 that has recently been shown to be required for
binding of Clb5/Cdc28 (Verma et al., 1997a) (Fig. 10).

Fig. 6. Overexpression of rum1+ in budding yeast induces a cell
cycle block in G1. The S. cerevisiae W303 strain was transformed
with the plasmids YipG3-SIC1 and YipG3-rum1+. Integrants were
selected in minimal medium with glucose and confirmed by Southern
blot. FACS analysis of cells overproducing SIC1 (A) or rum1+ (B) at
different times after the addition of galactose.

Fig. 7. Phenotype of budding yeast cells overexpressing rum1+.
Cultures from integrant-GALI-SIC1 (A,B) and integrant-GALI-
rum1+ (C,D) growing in glucose (A,C) or 8 hours after the addition
of galactose (B,D).

Fig. 8. Expression of rum1+ restores
the G1 arrest of cdc4-1 sic1Δ at
37°C. S. cerevisiae 15 Dau cdc4-1
sic1Δ cells were transformed with the
plasmids YipG2-SIC1 and YipG2-
rum1+. Integrants were selected in
minimal medium with glucose and
confirmed by Southern blot. Cells
were grown in YP raffinose at 25°C.
Galactose was added to a final
concentration of 2.5% and incubated
at 25°C to induce a transient
expression of rum1+ and SIC1 genes.
After 150 minutes, the cells were
centrifugated and resuspended in
YPD medium at 25°C or at 37°C.
DISCUSSION

Our results show that p25 rum1 and p40 Sic1 are structurally and functionally related Cdk inhibitors. Furthermore, expression of Sic1 rescues the phenotypes of the deletion of rum1 gene in fission yeast and overproduction of Sic1 in fission yeast also induces re-replication, suggesting that p40 Sic1 like p25 rum1 has specificity for the inhibition of S-phase and mitotic Cdk/cyclin complexes in fission yeast. None of the other Cdk inhibitors used in this study (budding yeast Far1 and animal cells p21 CIP1, p27 KIP1 and p16 INK4) were able to rescue the phenotype of the rum1 deletion nor did they generate any significant phenotype when overexpressed in fission yeast. S. cerevisiae Far1 inhibits only the G1 Cdc28/Cln complexes (Peter and Herskowitz, 1994), and animal cells p16 INK4a and p21 CIP1 and p27 KIP1 are known to inhibit preferentially the Cdk/cyclin complexes with

Fig. 9. Mapping of the cdc13 interacting domain and the Cdk inhibitory domain in p25 rum1
(A) Over-production of a truncated form of p25 rum1 from residues 67 to 147 is sufficient to block the cell cycle in fission yeast. A h− leu1-32 strain (Sp20) was transformed with pREP3X-rum1-52-147, pREP3X-rum1-67-147 and pREP3X-rum1-84-147 and grown in the presence (+ Th) or in the absence (− Th) of thiamine for 20 hours. (B) p25 rum1 interacts with cdc13 in the two hybrid system. pAS2 vectors containing different deletions of the rum1 gene were co-transformed with the pACT2-cdc13 vector. Transformants containing both plasmids were selected in minimal medium and β-galactosidase activity was measured.

Fig. 10. Amino acid sequence alignment of p25 rum1 and p40 Sic1. Identical residues in both proteins are indicated with an asterisk (*). The p25 rum1 Cdk inhibitory domain (residues 67 to 147) is included in the box.
a role in G1 or S-phase (Harper et al., 1995). The Cdk inhibitors described so far in animal cells have a low affinity for the mitotic Cdc2/cyclinB kinase complexes (Elledge et al., 1996; Harper et al., 1995), suggesting that either animal cells have evolved in such a way that they do not need a rum1/Sic1 homologue or that this homologue is still to be discovered.

There are many parallels between the function and the regulation of p25rum1 and p40SIC1. First, they both have a specificity for S-phase and mitotic Cdk/cyclin complexes. In budding yeast, p40SIC1 has been shown to inhibit specifically Cdc28/Cln associated kinases, preventing the initiation of S-phase and mitosis during G1 (Schwob et al., 1994). In fission yeast, p25rum1 inhibits cdc2/cig2 and cdc2/cdc13 complexes (Benito et al., 1998; Correa-Bordes and Nurse, 1995; Martin-Castellanos et al., 1996). Second, p40SIC1 is stabilised during anaphase and is degraded at the end of G1 through the Cdc4-Stern and Nurse, 1996; Wuarin and Nurse, 1996). At the end complexes (pre-RC) (Dahmann et al., 1995; Nasmyth, 1996; Castellanos et al., 1996). Second, p40SIC1 is stabilised during (Benito et al., 1998; Correa-Bordes and Nurse, 1995; Martín-Jallepalli and Kelly, 1996; Labib et al., 1995; Nasmyth, 1996). A similar mechanism appears to regulate the stability of the G1 cyclins Cln2 and Cln3 in S. cerevisiae (Deshaies et al., 1995; Lanker et al., 1996; Willems et al., 1996; Yaglom et al., 1995).

p25rum1 and p40SIC1: a common function in inhibiting Cdk activity during G1

p25rum1 and p40SIC1 play an important function in the G1 phase of the fission yeast and budding yeast cell cycle (Bai et al., 1996; Correa-Bordes and Nurse, 1995; Donovan et al., 1994; Jallepalli and Kelly, 1996; Labib et al., 1995; Martin-Castellanos et al., 1996; Moreno and Nurse, 1994; Nugroho and Mendenhall, 1994; Schwob et al., 1994; Schneider et al., 1996). Both proteins oscillate during the cell cycle being present from anaphase until the end of G1 (Benito et al., 1998; Donovan et al., 1994; Schwob et al., 1994). This pattern is the exact opposite of the pattern of cyclin B. During anaphase when Cib1-6 and cdc13 proteolysis takes place, p40SIC1 and p25rum1 begin to accumulate. Recently it has been shown that p25rum1 associates with the cdc2/cdc13 complex during G1 and is necessary to promote cdc13 degradation (Correa-Bordes et al., 1997). Proteolysis of mitotic cyclins in G1 is important to keep low Cdk/cyclin B activity in G1 cells, ensuring that S-phase initiation is coordinated with cell size and with other cell cycle events such as budding in S. cerevisiae.

It has been argued that inhibition of Cdc2/cyclin B complexes in G1 cells may be necessary to establish DNA pre-replication complexes (pre-RC) (Dahmann et al., 1995; Nasmyth, 1996; Stern and Nurse, 1996; Wuarin and Nurse, 1996). At the end of G1, phosphorylation of p40SIC1 in S. cerevisiae and p25rum1 in fission yeast by Cdc28/Cln and cdc2/cig1 kinase activity, respectively, target them for degradation and allow the switch from a state of low Cdk/cyclin B kinase activity to a state of high Cdk/cyclin B kinase activity (Benito et al., 1998; Verma et al., 1997b), which triggers the DNA replication process. Cdk/cyclin B complexes do not only trigger initiation from origins that have formed pre-RC but also prevent the de novo assembly of pre-RC (Dahmann et al., 1995). Thus, alteration of these two states of low and high Cdk/cyclin B kinase activity allows for the correct assembly of proteins at the DNA replication origin and that origins are fired only once per cell cycle.

We thank Cristina Martín-Castellanos for the data shown in Fig. 1A; Tamar Enoch and Karim Labib for comments to the manuscript; Michael Mendenhall for the sequence alignment between p25rum1 and p40SIC1 shown in Fig. 10; and Michael Mendenhall, Manuel Serrano, David Beach, Etienne Schwob, Kim Nasmyth, Korenli Polyak, Joan Massagué, Randy Poon, Steve Elledge and Mathias Peter for the gift of plasmids and strains. A.S.-D. is a fellow of the CSIC-Glaxo predoctoral fellowship program. This work was supported by the DGCYCT, the Human Frontier Science Program and the Ramón Areces Foundation.

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