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Fission yeast Fizzy-related protein srw1p is a G_1-specific promoter of mitotic cyclin B degradation

Satoko Yamaguchi[1,2], Hiroto Okayama[2] and Paul Nurse[1,3]

[1]Cell Cycle Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, UK and [2]Department of Biochemistry and Molecular Biology, The University of Tokyo, Graduate School of Medicine, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

[3]Corresponding author
e-mail: P.Nurse@icrf.icnet.uk

Downregulation of cyclin-dependent kinase (Cdk)–mitotic cyclin complexes is important during cell cycle progression and in G_1 arrested cells undergoing differentiation. srw1p, a member of the Fizzy-related protein family in fission yeast, is required for the degradation of cdc13p mitotic cyclin B during G_1 arrest. Here we show that srw1p is not required for the degradation of cdc13p during mitotic exit demonstrating that there are two systems operative at different stages of the cell cycle for cdc13p degradation, and that srw1p is phosphorylated by Cdk–cdc13p only becoming dephosphorylated during G_1 arrest. We propose that this phosphorylation targets srw1p for proteolysis and inhibits its activity to promote cdc13p turnover.

Keywords: Cdk/fission yeast/Fizzy-related/G_1 arrest/mitotic cyclin B degradation

Introduction

In all eukaryotes, cyclin-dependent kinases (Cdks) play a central role both in the G_1–S and the G_2–M cell cycle transitions (reviewed by Nurse, 1990; Nigg, 1995). A single Cdk encoded by *cdc2*$^+$ in fission yeast regulates both transitions. It is regulated during the cell cycle by several mechanisms including binding to a cyclin subunit, phosphorylation and Cdk inhibitors. A mitotic cyclin B encoded by *cdc13*$^+$ is essential for G_2–M progression (Booher and Beach, 1988; Hagan *et al*., 1988; Booher *et al*., 1989; Moreno *et al*., 1989). Cig2p acts as the major G_1–S cyclin (Obara-Ishihara and Okayama, 1994; Martin-Castellanos *et al*., 1996; Mondesert *et al*., 1996), although cdc13p can promote G_1–S progression in the absence of cig2p (Fisher and Nurse, 1996; Mondesert *et al*., 1996). Regulation of the cdc13p major mitotic cyclin is important during cell cycle progression. Cells deleted for cdc13p skip mitosis and undergo extra rounds of replication (Hayles *et al*., 1994), whilst expression of an indestructible *cdc13* blocks exit from mitosis (Yamano *et al*., 1996). Regulation of the activity of Cdks is also important when cells undergo differentiation. Fission yeast cells that are nitrogen starved or treated with pheromone arrest in G_1 and undergo conjugation and meiosis. These processes require downregulation of cdc2p–B-type cyclin complexes; pheromone induces G_1 arrest by inhibiting cdc2p–cdc13p and cdc2p–cig2p (Stern and Nurse, 1997) due to the degradation of cdc13p and cig2p and the action of the Cdk inhibitor rum1p (Stern and Nurse, 1998).

Mutants with defects in G_1 arrest and differentiation have been identified, some of which are involved in regulating Cdk–B-type cyclin activity. The *nuc2*$^+$ and *apc10*$^+$ gene products are components of the anaphase promoting complex/cyclosome (APC/C) and are conserved among eukaryotes (Hirano *et al*., 1988; Kumada *et al*., 1995; Kominami *et al*., 1998). Mutants in the *nuc2*$^+$ and *apc10*$^+$ genes undergo mitotic arrest at their restrictive temperature because they are unable to degrade mitotic cyclin. At their permissive temperature these mutants fail either to arrest in G_1 or to undergo conjugation. Studies of these mutants support the idea that downregulation of B-type cyclins is important both in the cell cycle and in cells undergoing differentiation, although it is not known whether the same system operates for the degradation of cdc13p at mitotic exit as well as during G_1 arrest in differentiating cells. In contrast to *nuc2* and *apc10*, both *rum1* and *srw1*/*ste9* mutants have no mitotic defects, although they fail to arrest in G_1 or to perform conjugation after nitrogen starvation (Moreno and Nurse, 1994; Yamaguchi *et al*., 1997; Kitamura *et al*., 1998). *rum1*$^+$ was isolated as a gene whose overexpression induces extra rounds of replication (Moreno and Nurse, 1994). It is a Cdk inhibitor that directly binds to Cdk–B-type cyclin (Correa-Bordes and Nurse, 1995; Jallepalli and Kelly, 1996; Martin-Castellanos *et al*., 1996) and is also required for the degradation of cdc13p during G_1 (Correa-Bordes *et al*., 1997). *srw1*$^+$/*ste9*$^+$ encodes a Fizzy-related protein that was initially isolated as a multicopy suppressor of hyperactive cdc2p–cdc13p complexes and is also required for the degradation of cdc13p during G_1 (Yamaguchi *et al*., 1997; Kitamura *et al*., 1998).

In budding yeast the degradation of mitotic cyclins is negatively regulated by *CDC28* (the budding yeast *cdc2*$^+$ homologue) (Amon *et al*., 1994), and the highly conserved WD repeat proteins Fizzy and Fizzy-related, which activate protein degradation, are important in this regulation. *Drosophila fizzy*, budding yeast *CDC20* and fission yeast *slp1*$^+$ are all required for the degradation of anaphase inhibitors during the metaphase–anaphase transition (Sethi *et al*., 1991; Dawson *et al*., 1995; Sigrist *et al*., 1995; Matsumoto, 1997; Visintin *et al*., 1997; Shirayama *et al*., 1998), while *Drosophila fizzy-related*, budding yeast *HCT1*/*CDH1* and fission yeast *srw1*$^+$/*ste9*$^+$ are required for the degradation of mitotic cyclins mainly during G_1 (Schwab *et al*., 1997; Sigrist and Lehner, 1997; Visintin *et al*., 1997; Yamaguchi *et al*., 1997; Kitamura *et al*., 1998). In budding yeast, Hct1 activity is negatively regulated by Cdc28 and phosphorylation inhibits

interaction between Hct1 and APC (Zachariae *et al*., 1998; Jaspersen *et al*., 1999).

To understand better the regulation of Cdk–B-type cyclin complex during the mitotic cell cycle and differentiation, we have studied the Fizzy-related protein srw1p function and its regulation in fission yeast. We show that srw1p is not required for cdc13p degradation at mitotic exit demonstrating that there are at least two systems operative for the degradation of cdc13p. We also show that srw1p is phosphorylated during the cell cycle by the cdc2p–cdc13p protein kinase, and that phosphorylation of srw1p by cdc2p affects the stability of srw1p and inhibits its activity to promote cdc13p turnover.

Results

srw1p is not required for cdc13p degradation at mitotic exit

srw1p is required for the degradation of the major fission yeast mitotic cyclin cdc13p when cells are arrested in G_1 and this degradation is necessary to prevent further progression through the cell cycle in these circumstances (Correa-Bordes *et al*., 1997; Yamaguchi *et al*., 1997; Kitamura *et al*., 1998; Kominami *et al*., 1998; Stern and Nurse, 1998). However, *srw1*Δ mutants are fully viable and show no mitotic defects, suggesting that srw1p may not have a role in the degradation of cdc13p, which is generally required for mitotic exit. To investigate this further, cells were synchronized at the G_2–M boundary by incubating the temperature-sensitive mutant *cdc25-22* at its restrictive temperature, 36°C. They were then released into mitosis, G_1 and S-phase by shifting down to 25°C. A strain with srw1p (*cdc25-22*) and one lacking srw1p (*cdc25-22 srw1*Δ) both underwent mitosis, septation (Figure 1A) and S-phase (Figure 1B) with the same kinetics. In wild-type cells, cdc13p becomes degraded at mitotic exit (Figure 1C) (Moreno *et al*., 1989). In *srw1*Δ cells, the degradation of cdc13p and tyrosine dephosphorylation of cdc2p occurred to the same extent and with similar kinetics to wild-type cells, as did the appearance of the G_1 marker rum1p (Figure 1C). This experiment demonstrates that srw1p has no influence on either the timing of mitotic exit or the kinetics of cdc13p degradation at the mitosis to G_1 transition. Therefore, we conclude that there are at least two systems operative for the degradation of cdc13p. One is required for cdc13p degradation at mitotic exit and is independent of srw1p, and the second is required to regulate cdc13p levels when cells are arrested in G_1 and this degradation system is dependent upon srw1p.

srw1p activity is correlated with its state of phosphorylation

The fact that srw1p has no role at mitotic exit and that its ectopic expression induces G_1 arrest suggests that srw1p activity may be maintained at a low level in rapidly growing cells. Antibodies raised against the entire protein produced in bacteria were used to show that the 62 kDa srw1p was present in exponentially growing cells (Figure 2A) and was found at all stages of the cell cycle in a *cdc25-22* block and release synchronized culture (Figure 2B). Thus, srw1p activity cannot be regulated during the cell cycle by maintaining its amount at a low level.

Several migration forms could be detected during gel electrophoresis (Figure 2A and C), and treatment with λ phosphatase resulted in the slowest migrating forms disappearing and the fastest forms increasing in amount (Figure 2C), indicating that srw1p is phosphorylated in proliferating cells. To test whether srw1p activity is correlated with its state of phosphorylation, the phosphorylation state of srw1p was monitored in cells arrested in G_1 where srw1p has been shown to be required for cdc13p degradation. In fission yeast, the G_1 phase of the cell cycle is short in rapidly growing cells. When starved for nitrogen, cells arrest in pre-Start G_1 in preparation for differentiation, and G_1 arrest can also be brought about by using *cdc10* mutants that arrest in pre-Start G_1. The *cdc10-129* mutant was used, which blocks cells in G_1 when incubated for 4 h at 36°C (Figure 3A). By this time point srw1p was observed to have become dephosphorylated (Figure 3B), cdc13p had disappeared from the cells, and cdc2p had become tyrosine dephosphorylated. In contrast, cdc13p and cdc2p tyrosine phosphorylation persisted in cells deleted for srw1p (Figure 3B), even though these cells eventually arrested in G_1 (Figure 3A). When wild-type cells were arrested in G_1 in response to nitrogen starvation, srw1p also became dephosphorylated and cdc13p disappeared (data not shown). As previously shown, in cells deleted for srw1p, cdc13p remained high even when cells became arrested in G_1 (Yamaguchi *et al*., 1997; Kitamura *et al*., 1998). Thus, srw1p becomes dephosphorylated in cells arrested in G_1, and srw1p is required for cdc13p degradation. At all stages of the cell cycle the mobility of srw1p was unchanged (Figure 2B), indicating that its phosphorylation state probably remains constant in rapidly growing cells. Mobility was retarded compared with the dephosphorylated form of srw1p found in blocked *cdc10-129* cells (Figure 2B), showing that srw1p is phosphorylated throughout the cell cycle. We conclude that srw1p is phosphorylated throughout the cell cycle and becomes dephosphorylated during G_1 arrest when srw1p is active.

srw1p phosphorylation is cdc2p dependent

During G_1 arrest when srw1p is dephosphorylated, the activity of the cdc2p Cdk is also much reduced. To investigate whether srw1p phosphorylation is dependent upon the cdc2p Cdk, we monitored the mobility state of srw1p in the temperature-sensitive *cdc2-M26* mutant. On shift to 36°C, srw1p became dephosphorylated (Figure 4A) even though the cells were mostly arrested in G_2, when srw1p would normally be phosphorylated. Next, we assessed the requirement of cdc2p for srw1p phosphorylation in cells arrested with hydroxyurea (HU) after the G_1–S transition. srw1p was phosphorylated in *cdc2-M26* mutant cells at 25°C arrested with HU, but became dephosphorylated when cdc2p was inactivated by shifting the cells to 36°C (Figure 4B). H1 kinase activity was monitored to determine the level of cdc2p kinase activity in the presence of HU. As previously reported (Knudsen *et al*., 1996; Rhind and Russell, 1998), cdc2p kinase activity was detected in cells treated with HU and was reduced when *cdc2-M26* mutant cells were shifted to 36°C (Figure 4B). Although the srw1p mobility shift was not as

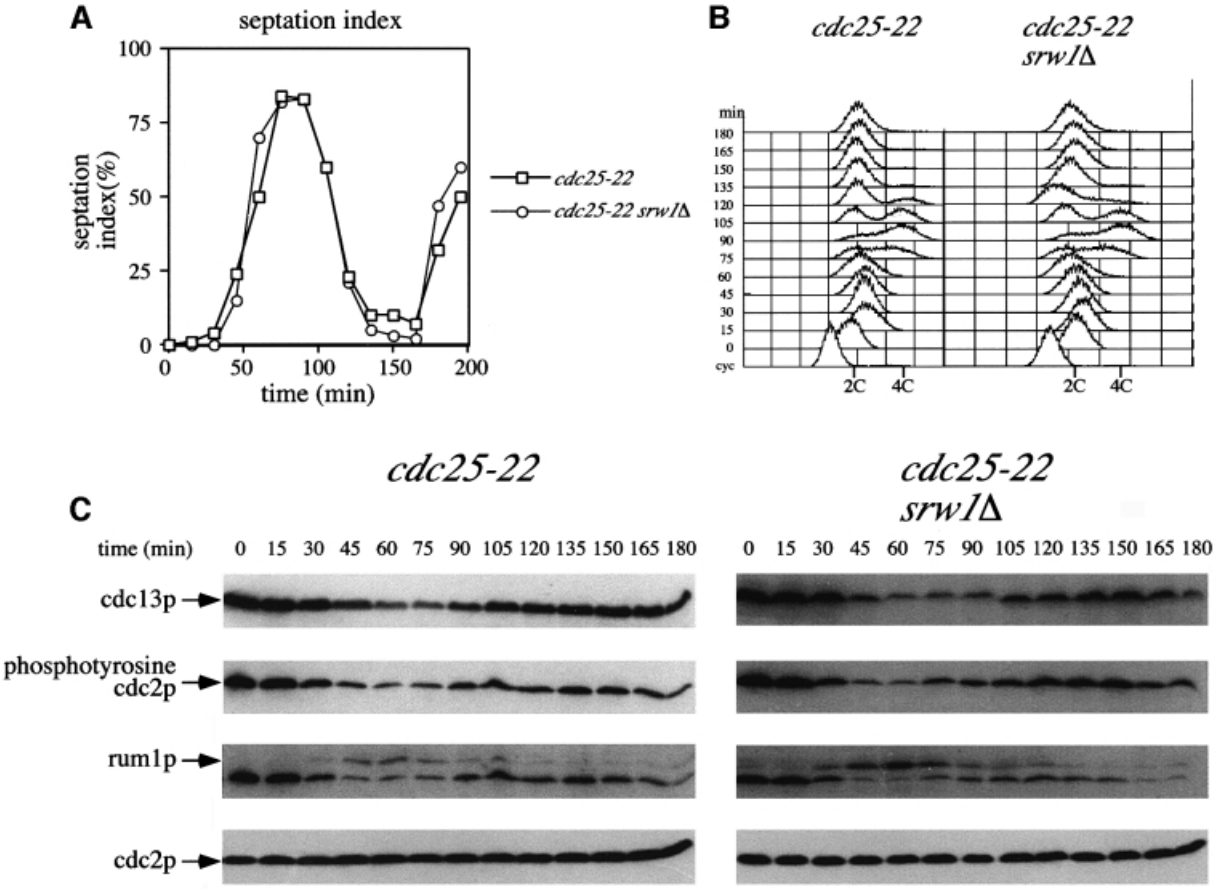

Fig. 1. srw1p is not required for cdc13p degradation at mitotic exit. (**A**) Septation index and (**B**) flow cytometric analysis of G_2 block and release experiments. *cdc25-22* and *cdc25-22 srw1*Δ strains were grown to exponential phase at 25°C (cyc), blocked for 4 h at 36°C, released at 25°C and incubated for the time indicated. (**C**) Western blots of the experiments described above using anti-cdc13p, anti-phosphotyrosine cdc2p, anti-rum1p and anti-cdc2p antibodies. For anti-rum1p antibody blotting, the same blots were used after the anti-phosphotyrosine cdc2p antibody.

great as when cells were shifted in the absence of HU, a clear difference between 36 and 25°C was detected (Figure 4B). This experiment eliminates effects of the cell cycle stage on phosphorylation and establishes that srw1p phosphorylation is at least partly dependent *in vivo* upon cdc2p activity.

cdc2p forms Cdk complexes with several cyclins, the major ones being cdc13p (Booher and Beach, 1988; Hagan *et al*., 1988; Booher *et al*., 1989; Moreno *et al*., 1989) and cig2p (Obara-Ishihara and Okayama, 1994; Martin-Castellanos *et al*., 1996; Mondesert *et al*., 1996), and a minor one, cig1p (Bueno *et al*., 1991). To determine which of these Cdks might be responsible for *in vivo* srw1p phosphorylation, srw1p mobility was monitored in mutant strains that lacked these cyclins. cdc13p activity was removed using both a *cdc13* switch off strain and a temperature-sensitive *cdc13-R9* mutant, and in both cases srw1p became dephosphorylated (Figure 4C). In contrast, in *cig1*Δ and *cig2*Δ mutant strains, srw1p remained phosphorylated (Figure 4D). These experiments establish that the cdc2p–cdc13p mitotic Cdk is largely responsible, either directly or indirectly, for *in vivo* phosphorylation of srw1p.

The conclusion that srw1p phosphorylation is dependent upon the cdc2p–cdc13p Cdk is consistent with the cell cycle timing of srw1p phosphorylation, as shown by monitoring srw1p mobility in *cdc10-129* mutant cells released synchronously into the cell cycle after a block at 36°C (Figure 5A). S-phase was observed at ~90 min after release, as monitored either by flow cytometry (Figure 5A) or by the disappearance of the rum1p G_1 marker (Figure 5C). At 90 min srw1p also became phosphorylated, and this phosphorylation was correlated with the appearance of cdc13p, the tyrosine phosphorylation of cdc2p (Figure 5C) and the appearance of cdc2p Cdk activity (Baum *et al*., 1997). Therefore, as cdc2p–cdc13p Cdk activity appears in the cells, srw1p becomes phosphorylated. In a *srw1*Δ mutant, cdc13p was still found to be present (Figure 5C) and as a consequence S-phase could not be initiated (Figure 5A). In contrast, when *srw1*Δ mutant cells were starved for nitrogen, they failed to arrest in G_1 (Yamaguchi *et al*., 1997; Kitamura *et al*., 1998; Kominami *et al*., 1998). This is expected because initiation of DNA replication occurs in two steps, the first of which is only possible if cdc2p Cdk activity is low and the second requires cdc2p Cdk activity (reviewed by Stern and Nurse, 1996). If Cdk activity is already high, as in this *cdc10-129* experiment, then S-phase would not be initiated because the first step requiring low Cdk activity would be unable to take place. Eventually the *srw1Δ* mutant cells enter mitosis and generate a 'cut' phenotype losing viability (Figure 5A and B), as previously reported (Kitamura *et al*., 1998).

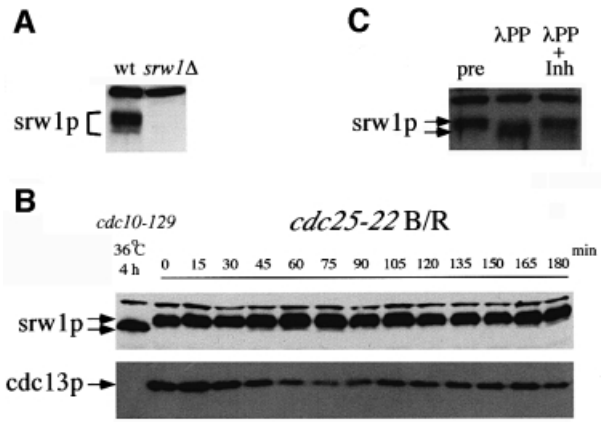

Fig. 2. srw1p is phosphorylated throughout the cell cycle. (**A**) Extracts were prepared from exponentially growing wild-type (wt) cells and *srw1Δ* cells, western blotted and probed with anti-srw1p antibody. The upper band is a non-specific cross-reacting protein. (**B**) *cdc25-22* strain was blocked and released (B/R) as described in Figure 1. Samples were western blotted and probed with anti-srw1p and anti-cdc13p antibodies. An extract from G_1 arrested *cdc10-129* cells was loaded as a control. (**C**) Extracts were prepared from exponentially growing wild-type cells (pre) and incubated with λ phosphatase in the absence (λPP) or presence (λPP + Inh) of phosphatase inhibitors.

This establishes the importance of srw1p in the proper regulation of the cdc2p–cdc13p Cdk during G_1 arrest, and during entry into the subsequent S-phase.

srw1p stability is regulated by phosphorylation on Cdk consensus sites

Examination of the srw1p protein sequence identified four Cdk consensus sites suggesting that it may be phosphorylated directly by the cdc2p Cdk. This was shown to be the case *in vitro* by adding immunoprecipitates of cdc2p and cdc13p to glutathione *S*-transferase (GST)–srw1p fusion protein as a substrate in a Cdk assay (Figure 6). Both immunoprecipitates contained an activity that could phosphorylate srw1p *in vitro*.

To confirm that srw1p is phosphorylated on these sites *in vivo*, a quadruple mutant of the *srw1* gene was constructed with alanine substitutions at all four of the Cdk consensus sites (S62, S214, T98, T177). When the quadruple mutant was expressed *in vivo*, the mobility of srw1p was increased, demonstrating that phosphorylation was occurring at the Cdk consensus sites (Figure 7A). cdc2p phosphorylation can bring about the degradation of substrates such as cdc18p and rum1p (Jallepalli *et al*., 1997; Baum *et al*., 1998; Benito *et al*., 1998), and so the effect of cdc2p phosphorylation on srw1p stability was also tested. Switch off strains of wild-type srw1p and the quadruple phosphorylation mutant of srw1p using the *nmt81* promoter were constructed for these experiments. When the promoter was switched on, the levels of both wild-type srw1p and mutant srw1p were no more than four times the endogenous level of srw1p. Upon switch off, the wild-type srw1p disappeared with a half-life of ~40 min, whilst the quadruple mutant was much more stable with a half-life of >120 min (Figure 7B). We conclude that phosphorylation of srw1p on its Cdk consensus sites targets the protein for degradation. To investigate whether srw1p stability is regulated by phosphorylation, we tested

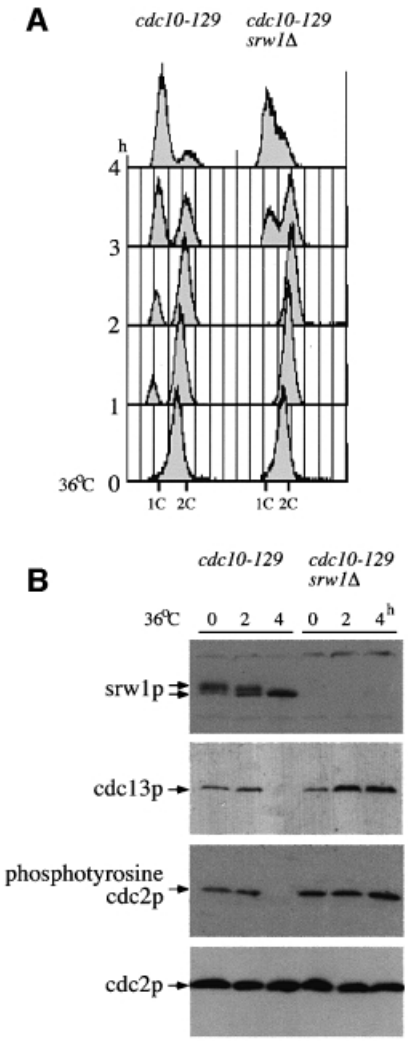

Fig. 3. srw1p becomes dephosphorylated during G_1 arrest, which coincides with the degradation of cdc13p. (**A**) Flow cytometry of G_1 block experiments. *cdc10-129* and *cdc10-129 srw1Δ* strains were shifted to 36°C and incubated for the indicated time in hours. (**B**) Western blots of the experiments above using anti-srw1p, anti-cdc13p, anti-phosphotyrosine cdc2p and anti-cdc2p antibodies.

the stability of srw1p in rapidly growing cells and in G_1 blocked cells, and compared the stability of phosphorylated and dephosphorylated srw1p. After incubating wild-type cells and *cdc10-129* cells at 36°C for 4 h, cycloheximide was added to inhibit protein synthesis (Ayscough and Warren, 1994; Jallepalli *et al*., 1997). In the wild-type cells, srw1p was mostly phosphorylated and disappeared quickly after adding cycloheximide with a half-life of 30 min, whilst in the *cdc10-129* cells, srw1p was dephosphorylated and more stable with a half-life of >120 min (Figure 7C). This confirms that srw1p stability is regulated *in vivo* by phosphorylation.

When cells were grown continually in the presence of the srw1p quadruple mutant, diploid cells were found to accumulate in the culture (Figure 7D), which was not observed when wild-type *srw1*$^+$ was expressed under the same conditions (data not shown), indicating that phosphorylation of srw1p by cdc2p is important for genomic stability. Increasing the levels of cdc13p suppressed this effect (Figure 7D), suggesting that it might be a reduction

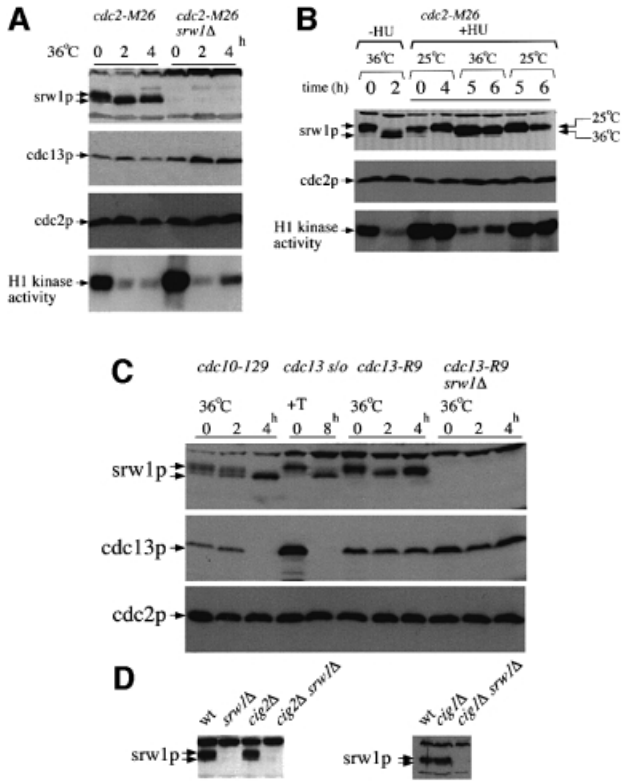

Fig. 4. srw1p phosphorylation is cdc2p dependent. (**A**) *cdc2-M26* and *cdc2-M26 srw1*Δ strains were shifted to 36°C and incubated for the indicated time in hours. Extracts were western blotted and probed with anti-srw1p, anti-cdc13p and anti-cdc2p antibodies. Extracts were immunoprecipitated with anti-cdc2p antibody, and H1 kinase activity of each immunoprecipitate was measured. (**B**) *cdc2-M26* strain was blocked with 11 mM HU for the indicated time in hours. Further 11 mM HU was added after 4 h to prevent cells from leaking from the block. Cells were incubated for 4 h at 25°C and then incubated for another 2 h at either 36 or 25°C. Extracts from *cdc2-M26* cells blocked in the absence of HU were also loaded. The arrows indicate the migration of srw1p in experiments at 36 and 25°C, respectively. Extracts were immunoprecipitated with anti-cdc2p antibody, and H1 kinase activity of each immunoprecipitate was measured. (**C**) *cdc13* was shut off by adding thiamine (+T) to the *cdc13*Δ *REP45-cdc13*$^{+}$ strain (*cdc13 s/o*) and cells were incubated at 30°C for the indicated time in hours. *cdc13-R9* and *cdc13-R9 srw1*Δ strains were shifted to 36°C and incubated for the indicated time in hours. Extracts from *cdc10-129* cells were loaded as controls. Extracts were western blotted and probed with anti-srw1p, anti-cdc13p and anti-cdc2p antibodies. (**D**) Extracts were taken from exponentially growing wild-type (wt), *srw1*Δ, *cig2*Δ, *cig2*Δ *srw1*Δ, *cig1*Δ and *cig1*Δ *srw1*Δ cells, western blotted and probed with anti-srw1p antibody.

of endogenous cdc13p levels caused by the constitutive srw1p activity that was responsible for the observed diploidization. To test whether there is an increased activity of srw1p in the quadruple mutant, cdc13p turnover was examined using a *cdc13* switch off strain. Upon switch off, cdc13p was degraded more quickly in the quadruple mutant than in wild type (Figure 7E). These results suggest that phosphorylation of srw1p inhibits its activity to promote cdc13p turnover.

Discussion

Earlier work has shown that degradation of the cdc13p mitotic cyclin occurs at two phases of the fission yeast cell cycle, both at mitotic exit and during G_1 arrest (Moreno *et al*., 1989; Hayles and Nurse, 1995; Correa-Bordes *et al*., 1997). In this paper we have shown that the mechanisms bringing about the degradation of cdc13p mitotic cyclin B differ at these two different phases of the cell cycle. During G_1 arrest, degradation is dependent on the WD repeat Fizzy-related protein srw1p (Yamaguchi *et al*., 1997; Kitamura *et al*., 1998; Figure 3) but this protein is not required for the degradation of cdc13p at exit from mitosis (Figure 1). We have also shown that srw1p is phosphorylated by Cdk and that this phosphorylation targets srw1p for proteolysis.

Fizzy-related proteins are highly conserved in eukaryotes, including Hct1/Cdh1 in budding yeast, Fizzy-related in *Drosophila* and Cdh1 in mammalian cells (Schwab *et al*., 1997; Sigrist and Lehner, 1997; Visintin *et al*., 1997; Fang *et al*., 1998; Kramer *et al*., 1998). In fission yeast, both srw1p and the Cdk inhibitor rum1p are required during G_1 arrest and in cells differentiating from the G_1 phase of the cell cycle, and the double deletion mutant of *srw1* and *rum1* is fully viable and shows no mitotic defects (our unpublished data; Kitamura *et al*., 1998). In *Drosophila*, the Fizzy-related protein is not found in cells undergoing rapid mitotic proliferation and is only observed later during development when cells develop an extended G_1 and begin differentiation (Sigrist and Lehner, 1997). *Fizzy-related* and Cdk inhibitor *rux* mutants both undergo an additional mitotic cycle before arresting in G_1 prior to differentiation (Gonczy *et al*., 1994; Thomas *et al*., 1994; Sigrist and Lehner, 1997). These results indicate that these *Drosophila* mutant cells have difficulty in maintaining G_1 arrest, a phenotype which is similar to that of the fission yeast *srw1* and *rum1* mutants. In *Xenopus* embryos, the Fizzy-related FZR protein was also not detected until a G_1 phase appeared around the time of the mid-blastula transition (Lorca *et al*., 1998). Therefore, Fizzy-related proteins appear to be required for the degradation of mitotic cyclins in a number of eukaryotic cells undergoing G_1 arrest and differentiation.

There are two conserved members of WD repeat proteins in all eukaryotes that are involved in the degradation of APC substrates, Cdc20/slp1p/Fizzy/p55CDC (Sethi *et al*., 1991; Weinstein *et al*., 1994; Dawson *et al*., 1995; Sigrist *et al*., 1995; Matsumoto, 1997) and Hct1/srw1p/Fizzy-related/Cdh1 (Schwab *et al*., 1997; Sigrist and Lehner, 1997; Visintin *et al*., 1997; Yamaguchi *et al*., 1997; Fang *et al*., 1998; Kitamura *et al*., 1998; Kramer *et al*., 1998). In a number of these organisms the Fizzy and Fizzy-related protein families appear to act as specific cell cycle phase activators of mitotic cyclin degradation, with Fizzy acting during mitosis and Fizzy-related acting during G_1. Fizzy is required for the degradation of mitotic cyclins and for exit from mitosis in rapidly growing *Drosophila* (Sigrist and Lehner, 1997) and *Xenopus* (Lorca *et al*., 1998) cells that do not express Fizzy-related proteins. In budding yeast, Cdc20 and Hct1 had been thought to act as substrate-specific activators of APC, with Cdc20 activating the degradation of Pds1, and Hct1 activating the degradation of Clb2 (Schwab *et al*., 1997; Visintin *et al*., 1997; Shirayama *et al*., 1998). However, more recently it has been shown that Clb2 degradation in mitosis is dependent on Cdc20, not Hct1 (Yeong *et al*., 2000). In fission yeast, Fizzy protein slp1p

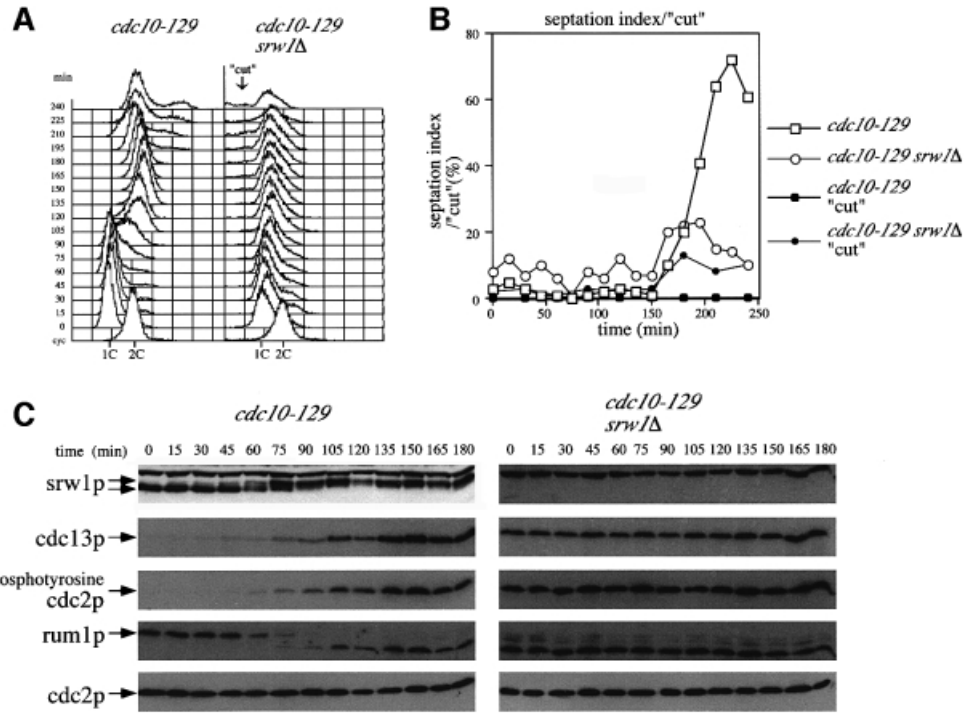

Fig. 5. srw1p becomes phosphorylated just before S-phase starts, which coincides with the accumulation of cdc13p. (**A**) Flow cytometry and (**B**) septation index and percentage of 'cut' cells in G_1 block and release experiments. *cdc10-129* and *cdc10-129 srw1*Δ strains were blocked for 4 h at 36°C and released into 25°C and incubated for the times indicated. (**C**) Western blots of the experiments described above using anti-srw1p, anti-cdc13p, anti-phosphotyrosine cdc2p, anti-rum1p and anti-cdc2p antibodies. For anti-rum1p antibody blotting, the same blots were used after the anti-phosphotyrosine cdc2p antibody.

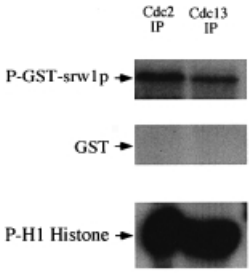

Fig. 6. srw1p is phosphorylated by cdc2p *in vitro*. cdc2p and cdc13p-associated cdc2p were immunoprecipitated from wild-type extracts and assayed for protein kinase activity using GST–srw1p or histone H1 as substrates. GST was used as a control.

activates the degradation of cut2p at the metaphase–anaphase transition, and so it is likely that slp1p is responsible for the degradation of mitotic cyclin at the exit from mitosis.

We have shown that srw1p is phosphorylated throughout the cell cycle, only becoming dephosphorylated during G_1 arrest (Figures 2 and 3) when srw1p is required for the degradation of cdc13p. srw1p phosphorylation requires the cdc2p protein kinase *in vivo* (Figure 4A and B), and *in vitro* kinase assays revealed that cdc2p can phosphorylate srw1p directly (Figure 6). Analysis of *srw1* phosphorylation-deficient mutants demonstrated that srw1p is phosphorylated at Cdk consensus sites *in vivo* (Figure 7A). Mutant forms of *srw1* that could not be phosphorylated on the four Cdk consensus sites in srw1p were more stable than wild-type srw1p: the half-life of the unphosphorylatable srw1p was >120 min, compared with 40 min for wild-type srw1p (Figure 7B). This establishes that cdc2p phosphorylation stimulates proteolysis of srw1p. Consistent with this view, when protein synthesis was inhibited, srw1p in G_1 arrested cells was more stable than srw1p in proliferating cells (Figure 7C). Although phosphorylation of srw1p affects its stability, srw1p levels did not change significantly during G_1 arrest when srw1p was dephosphorylated (Figures 3 and 5), suggesting that cdc2p-regulated proteolysis is not the only mechanism by which srw1p abundance is controlled. The unphosphorylatable *srw1* mutant causes diploidization (Figure 7D), indicating that the proper regulation of srw1p by cdc2p-mediated phosphorylation is required to maintain genomic stability. This effect was suppressed by increased levels of cdc13p (Figure 7D). Loss of cdc13p and overexpression of srw1p have both been shown to induce extra rounds of DNA replication (Hayles *et al*., 1994; Yamaguchi *et al*., 1997; Kitamura *et al*., 1998), probably by re-initiating S-phase in G_2 cells due to reduced cdc2p activity. The observed diploidization might be due to some effect on cdc13p caused by increased srw1p activity in unphosphorylatable *srw1* mutant cells. Consistent with this view, cdc13p was degraded more quickly in unphosphorylatable *srw1* mutant cells (Figure 7E), indicating that phosphorylation leads to the inhibition of srw1p activity. However, no significant difference in endogenous cdc13p levels was detected between unphosphorylatable *srw1* mutant cells and wild-type cells, indicating that there must be some other homeostatic mechanism operative that maintains cdc13p at a constant level. It is possible that cdc2p phosphorylation

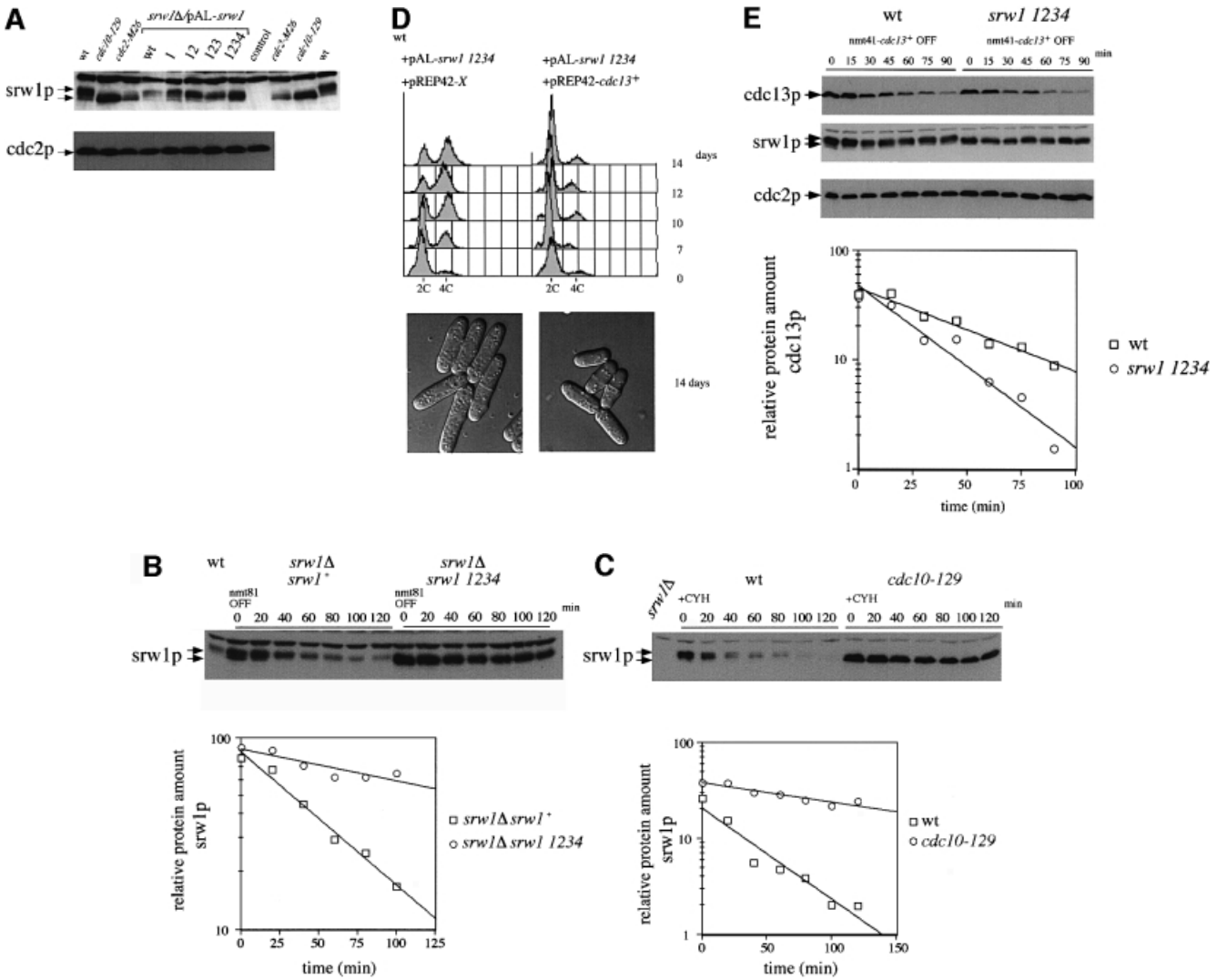

Fig. 7. srw1p stability is regulated by phosphorylation on Cdk consensus sites. (**A**) Samples were taken from exponentially growing *srw1*Δ cells carrying pAL-*srw1* with no (wt), single (1), double (12), triple (123) or quadruple (1234) mutations in Cdk consensus sites or empty vector (pAL-*X*). Extracts from wild-type cells, *cdc10-129* blocked cells and *cdc2-M26* blocked cells were loaded as controls. Extracts were western blotted and probed with anti-srw1p and anti-cdc2p antibodies. (**B**) *srw1* was shut off by adding thiamine (+T) to *srw1*Δ cells expressing wild-type *srw1* (*srw1*Δ *nmt81-srw1*$^+$) or quadruple mutant *srw1* (*srw1*Δ *nmt81-srw1 1234*) and cells were incubated at 30°C for the times indicated. Extracts from wild-type cells were loaded to show the endogenous level of srw1p. Extracts were western blotted and probed with anti-srw1p antibody, the srw1p protein was quantified with NIH image analyser. (**C**) Wild-type cells and *cdc10-129* cells were incubated for 4 h at 36°C, cycloheximide (CYH) was added, and cells were incubated at 36°C for the times indicated. (**D**) Flow cytometry and morphology of wild-type cells co-expressing quadruple mutant *srw1* (pAL-*srw1 1234*) and either pREP42-*X* or pREP42-*cdc13*$^+$. Cells were incubated for the number of days indicated at 30°C in minimal medium in the absence of thiamine. (**E**) *cdc13* expression was turned off by adding thiamine to *cdc13*Δ *REP41-cdc13*$^+$ and *cdc13*Δ *REP41-cdc13*$^+$ *srw1:: srw1 1234* strains and cells were further incubated at 32°C for the time indicated in minutes. Extracts were western blotted and probed with anti-cdc13p, anti-srw1p and anti-cdc2p antibodies.

of srw1p may have two regulatory effects: to stimulate the proteolysis of srw1p and to inhibit srw1p activity directly.

We have also shown that the phosphorylation of srw1p is mainly dependent on the cdc2p–cdc13p complex. srw1p became dephosphorylated when the mitotic cyclin cdc13p was inactivated but remained phosphorylated in mutants deleted for the G_1 cyclin *cig2* or *cig1* (Figure 4C and D), and *in vitro* kinase assays showed that the cdc13p-associated kinase could phosphorylate srw1p (Figure 6). The timing of srw1p phosphorylation at the G_1–S transition correlates with the accumulation of cdc13p in the cell (Figure 5). When cdc2p–cdc13p Cdk activity is low at mitotic exit or in a *cdc25* block (Moreno *et al*., 1989), srw1p is still phosphorylated (Figure 2B), suggesting that a low activity of cdc2p–cdc13p Cdk is sufficient to phosphorylate srw1p, and also that a phosphatase exists that brings about the dephosphorylation of srw1p during G_1 arrest. The phosphorylation of srw1p by the cdc2p–cdc13p complex might render srw1p inactive by targeting it for proteolysis and by directly inhibiting its activity so that the cdc13p level is sufficient for cells to proceed through the cell cycle. Such a regulatory mechanism has positive feedback characteristics providing a point of no return so that cells become committed to further cell cycle

Table I. *Schizosaccharomyces pombe* strains used in this study

Strain	Genotype
ATCC36399	*h⁻ leu1-32*
PN513	*h⁻ ura4-D18 leu1-32*
SY1	*h⁻ srw1Δ::ura4⁺ ura4-D18 leu1-32*
PN1419	*h⁻ cdc25-22 leu1-32*
SY15	*h⁻ cdc25-22 srw1Δ::ura4⁺ ura4-D18 leu1-32*
PN9	*h⁻ cdc10-129 leu1-32*
SY24	*h⁻ cdc10-129 srw1Δ::ura4⁺ ura4-D18 leu1-32*
PN30	*h⁻ cdc2-M26 leu1-32*
SY9	*h⁻ cdc2-M26 srw1Δ::ura4⁺ ura4-D18 leu1-32*
PN143	*h⁻ cdc13-R9 leu1-32*
SY16	*h⁻ cdc13-R9 srw1Δ::ura4⁺ ura4-D18 leu1-32*
PN1414	*h⁻ cdc13Δ::ura4⁺ ade6-704 leu1-32 ura4-D18 pREP45::cdc13⁺* (Hayles *et al*., 1994)
SY124	*h⁺ˢ cig2Δ::ura4⁺ ura4-D18 leu1-32*
SY5	*h⁻ cig2Δ::ura4⁺ srw1Δ::ura4⁺ ura4-D18 leu1-32*
SY127	*h⁻ cig1Δ::ura4⁺ ura4-D18 leu1-32* (Bueno *et al*., 1991)
SY21	*h⁺ˢ cig1Δ::ura4⁺ srw1Δ::ura4⁺ ura4-D18 leu1-32*
SY51	*h⁻ srw1Δ::ura4⁺ pREP81::srw1⁺ int ura4-D18 leu1-32*
SY52	*h⁻ srw1Δ::ura4⁺ pREP81::srw1 1234 int ura4-D18 leu1-32*
PN1260	*h⁺ cdc13Δ::ura4⁺ ura4-D18 leu1-32 pREP41::cdc13⁺ int*
SY60	*h⁻ cdc13Δ::ura4⁺ ura4-D18 leu1-32 pREP41::cdc13⁺ int srw1::srw1 1234*

progression. An interesting as yet unanswered question that will require further study is which occurs first, cdc13p accumulation or the phosphorylation of srw1p.

We conclude that the Fizzy-related protein srw1p is a G_1-specific promoter of mitotic cyclin cdc13p degradation. It plays no role in mitotic exit but is necessary to block cell cycle progression in cells arrested in G_1 and has an important role in ensuring proper sexual differentiation that occurs from cells arrested in the G_1 phase of the cycle. srw1p is phosphorylated by cdc2p–cdc13p Cdk activity and this phosphorylation targets srw1p for proteolysis. On exit from a G_1 block srw1p phosphorylation occurs as cdc2p–cdc13p Cdk activity increases in the cells. The involvement of Fizzy-related proteins in properly regulating G_1 arrest in differentiating cells may also be conserved in metazoan cells, given that these proteins have been shown to be necessary for proper G_1 regulation in *Drosophila* and *Xenopus* embryonic cells arrested in G_1 or undergoing differentiation from G_1.

Materials and methods

Fission yeast strains and methods

All experiments were carried out in EMM2 minimal media, and growth conditions are as described previously (Moreno *et al*., 1991). Strains used in this study are shown in Table I. Thiamine-repressible promoters *nmt45* and *nmt81* were used (Maundrell, 1990, 1993), with thiamine being used at 5 μg/ml. Cycloheximide was used at 100 μg/ml. Temperature-sensitive strains were grown exponentially at 25°C and shifted to 36°C, and other strains were grown at 30°C. Yeast transformation was performed as described previously (Bähler *et al*., 1998).

Purification of GST–srw1p

The *srw1⁺* gene was subcloned into pGEX5X-1 (Pharmacia) and the GST–srw1p fusion protein was produced in *Escherichia coli* carrying pGEX5X-1-*srw1⁺* and the protein purified with glutathione–Sepharose 4B (Pharmacia).

Anti-srw1p antibody

Purified GST–srw1p protein was used to raise anti-srw1p polyclonal antibodies. Anti-GST–srw1p serum was purified by blot affinity purification for western blotting.

Western blotting

Cells were boiled for 6 min after being washed once in Stop buffer (150 mM NaCl, 50 mM NaF, 1 mM NaN_3, 10 mM EDTA pH 8.0). Protein extracts were made by vortexing with glass beads in HB buffer (Moreno *et al*., 1991) and protein concentration determined with the BCA assay kit (Sigma). Cell extracts were re-boiled in 2× sample buffer and 50 μg of each sample were run in an 8% SDS–polyacrylamide gel (Laemmli, 1970). The protein was blotted to Immobilon™-P membrane (Millipore) and detected using ECL (Amersham). The antibodies used were purified anti-srw1p polyclonal antibody (1:200), anti-cdc13p monoclonal antibody 6F (1:500; made by Hayles and Steel), anti-phosphotyrosine cdc2p polyclonal antibody (1:1000; New England Biolabs), purified anti-rum1p polyclonal antibody R4 (1:500; Correa-Bordes and Nurse, 1995) and anti-cdc2p monoclonal antibody Y63 (1:100; Yamano *et al*., 1996).

Phosphatase treatment

Extracts were prepared from exponentially growing wild-type cells, followed by boiling. Cells were broken using glass beads and recovered in HB without any phosphatase inhibitors. λ phosphatase (New England Biolabs), λ phosphatase buffer (New England Biolabs) and 2 mM $MnCl_2$ were added, and the extracts were incubated at 30°C for 20 min with or without phosphatase inhibitors (60 mM β-glycerophosphate, 15 mM *p*-nitrophenylphosphate, 0.1 mM sodium vanadate). The reactions were stopped by the addition of 2× sample buffer before boiling for 3 min.

Flow cytometric analysis

Flow cytometric analysis was performed on a Becton-Dickinson FACScan using propidium iodide staining of cells as described previously (Sazer and Sherwood, 1990).

Phosphorylation experiments in vitro

Protein A and 5 μl of anti-cdc2p polyclonal antibody C2 (Simanis and Nurse, 1986) or protein G and 10 μl of anti-cdc13p monoclonal antibody 6F were rotated in HB buffer at room temperature for 30 min. After being washed three times with HB, these antibodies were rotated with 5 mg of cell extract at 4°C for 2 h. Immunoprecipitates were incubated with 200 μM ATP, 40 μCi/ml [γ-^{32}P]ATP, and 0.2 mg/ml GST–srw1p, 0.2 mg/ml GST (Sigma) or 1 mg/ml histone H1 (Calbiochem) at 30°C for 30 min. The reactions were stopped by boiling for 3 min after the addition of 2× SDS sample buffer and samples were analysed by 15% SDS–polyacrylamide gel.

Site-directed mutagenesis of Cdk phosphorylation consensus sites

The serine and threonine residues of the four Cdk phosphorylation consensus sites S/T-P-R-K (S62, S214, T98, T177) were changed to alanine using the Quickchange site-directed mutagenesis kit (Stratagene).

The mutated clones were confirmed by sequencing of the open reading frame. *srw1* shut off strains were constructed by transforming linearized pREP81-*srw1*$^+$ or pREP81-*srw1 1234* (quadruple mutant) into *srw1*Δ mutant. Cells were grown in rich medium and integrants were selected for leucine stability. For the *srw1::srw1 1234* strain, the 6 kb genomic fragment carrying the four mutations was transformed into *srw1*Δ cells selecting for 5-fluororotic acid resistance, and strain construction was confirmed by colony PCR.

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