Xe-p9, a *Xenopus* Suc1/Cks homolog, has multiple essential roles in cell cycle control

Deabrata Patra and William G. Dunphy

Division of Biology 216-76, Howard Hughes Medical Institute, California Institute of Technology, Pasadena, California 91125 USA

The small Suc1/Cks protein is a ubiquitous subunit of Cdk/cyclin complexes, but its precise function has remained unclear. We have isolated a *Xenopus* homolog, Xe-p9, of the Suc1/Cks protein by virtue of its ability to rescue a fission yeast mutant that enters mitosis prematurely. To assess its functional role in cell cycle control, we have both overexpressed p9 in *Xenopus* egg extracts and immunodepleted the protein from these extracts. We found that addition of recombinant His6-p9 to egg extracts results in a pronounced delay of mitosis that can be attributed to an inhibition of the tyrosine dephosphorylation of the inactive Cdc2/cyclin B complex. In immunodepletion studies, we observed that the consequences of removing p9 from egg extracts depend on the stage of the cell cycle. Specifically, in the case of interphase extracts, the removal of p9 abolishes the entry into mitosis as a result of a failure in the activation of the Cdc2/cyclin B complex by tyrosine dephosphorylation. Furthermore, mitotic extracts lacking p9 fail to exit mitosis because of a defect in the destruction of cyclin B. Collectively, these results indicate that p9 has multiple essential roles in the cell cycle by governing the interaction of the Cdc2/cyclin B complex with both positive and negative regulators.

[Key Words: Suc1 protein; Cdc2 protein kinase; mitosis; Cdc25; cyclin degradation; phosphotyrosine]

Received March 20, 1996; revised version accepted April 29, 1996.

The cell cycle consists of a series of highly coordinated steps that result in the production of two genetically identical daughter cells. It is essential that the cell cycle be regulated properly to maintain the genetic integrity of progeny cells. The precise coordination of cell division is achieved by the interplay of the cyclin-dependent kinases (Cdk) and a large repertoire of regulatory factors (for review, see Coleman and Dunphy 1994; Dunphy 1994; King et al. 1994; Nurse 1994; Morgan 1995). The Cdk's consist of a catalytic Cdk subunit whose kinase activity is dependent on a positive regulatory partner called cyclin. In yeasts, only a single Cdk catalytic subunit (Cdc28 in the budding yeast *Saccharomyces cerevisiae* or Cdc2 in the fission yeast *Schizosaccharomyces pombe*) is known to have a role in cell cycle progression. Yeast cells achieve the desired temporal control of the cell cycle by regulating this single Cdk subunit through the binding of different classes of cyclins in a sequential manner (for review, see Lew and Reed 1992; Nasmyth 1993). In contrast, vertebrate cells contain multiple Cdk subunits in addition to a diverse array of cyclins, which allows for combinatorial regulation of Cdk/cyclin complexes (for review, see Pines 1993; Hunter and Pines 1994; Sherr 1994; Sherr and Roberts 1995). The activation of Cdk/cyclin complexes in both yeast and animal cells is subject to stringent control to achieve an error-free execution of the cell division cycle.

In higher eukaryotes such as *Xenopus*, an important member of the Cdk family is the protein kinase Cdc2, which forms a complex with a B-type cyclin to form MPF or maturation-promoting factor (Dunphy et al. 1988; Gautier et al. 1988; Milarski et al. 1991). At the G2/M transition, the action of MPF results in the phosphorylation of numerous structural and regulatory proteins that are responsible for mitotic processes. The activation of MPF requires not only synthesis of the cyclin protein (Murray and Kirschner 1989; Solomon et al. 1990) but also involves post-translational modifications of the Cdc2 kinase on its Thr-161, Tyr-15, and Thr-14 residues. The phosphorylation of Cdc2 by the Cdk-activating kinase (CAK) on Thr-161 is essential for its activity (for review, see Draetta 1993; Solomon 1994; Morgan 1995). However, concomitant phosphorylations of Cdc2 on Tyr-15 and Thr-14 by the Wee1 and Myt1 protein kinases (Parker and Piwnica-Worms 1992; McGowan and Russell 1993; Atherton-Fessler et al. 1994; Kornbluth et al. 1994; Mueller et al. 1995a,b) keep MPF inactive throughout interphase. At the beginning of mitosis, the Cdc25 protein dephosphorylates Tyr-15 and Thr-14, resulting in the activation of MPF (Dunphy and Kumagai 1991; Gautier et al. 1991).

Genetic studies in yeast have revealed the existence of

---

1Corresponding author.
identified on the basis of its ability to suppress certain temperature-sensitive mutations of Cdc2 on overexpression in fission yeast [Hayles et al. 1986a,b]. Likewise, the budding yeast homolog Cks1 was identified as a suppressor of temperature-sensitive Cdc28 mutations [Hadwiger et al. 1989]. Both Sucl and Cks1 have been shown to interact physically with Cdc2 and Cdc28, respectively [Brizuela et al. 1987; Hadwiger et al. 1989]. However, a clear picture regarding the physiological function of this interaction has remained elusive. In fission yeast cells and *Xenopus* oocyte extracts, high concentrations of the Sucl protein delay the entry into the mitotic state [Hayles et al. 1986b; Dunphy et al. 1988]. However, the recombinant Sucl protein is clearly not a direct inhibitor of Cdk-associated kinase activity [Arion et al. 1988; Moreno et al. 1989]. Genetic disruption studies have indicated that Sucl and Cks1 are both essential for viability in fission and budding yeast [Hayles et al. 1986b; Hindley et al. 1987; Hadwiger et al. 1989]. Moreover, conditional mutations of Cks1 in budding yeast disrupt the ability of cells to undergo both the G1/S and G2/M transitions [Tang and Reed 1993]. Finally, cytological analysis of fission yeast in which the sucl gene had either been deleted or placed under the control of a repressible promoter indicated that Sucl-deficient cells cannot exit mitosis properly [Moreno et al. 1989; Basi and Draetta 1995]. In principle, this phenotype could result either from an impairment of cyclin degradation or the triggering of a checkpoint attributable to an inappropriate entry into mitosis. Taken together, these various findings appear to suggest that the Sucl/Cks1 protein may have multiple roles in cell cycle control, but clearly, further studies are required to reconcile these diverse experimental observations.

We have now cloned a *Xenopus* homolog (Xe-p9) of the Sucl/Cks1 family of proteins by using a frog oocyte cDNA library to rescue a mitotic control mutant of fission yeast. Using both recombinant p9 protein and anti-p9 antibodies, we have investigated the role of p9 in controlling the Cdc2/cyclin B complex in cell cycle extracts from *Xenopus* eggs. We find that p9 regulates both the activation of Cdc2/cyclin B at the G2/M boundary and the inactivation of this complex through degradation of cyclin B at the metaphase–anaphase transition. The multifaceted function of p9 helps to rationalize the diverse observations on the Sucl/Cks1 protein in earlier genetic and biochemical studies.

**Results**

**Isolation of Xe-p9, a Xenopus Sucl/Cks homolog**

To isolate *Xenopus* regulators of the cell cycle, our laboratory has been expressing a frog oocyte cDNA library [Mueller et al. 1995a; Carpenter et al. 1996] in a variety of fission yeast mitotic control mutants. In this study we transformed the cDNA library into the fission yeast strain *S. pombe rad3-136 wee1-50 ura4-D18 leu1-32 h*+ [Seaton et al. 1992]. This strain is not viable at the restrictive temperature of 35.5°C because of a synthetic lethality resulting from a defective Rad3 checkpoint control protein and a temperature-sensitive *Wee1* tyrosine kinase. One class of rescued strain that could grow at 35.5°C was found to harbor a plasmid containing an open reading frame (ORF) of 79 amino acids (9 kD) that displays strong homology to the Sucl/Cks family of proteins [Fig. 1A,B]. Among the members of this family in fission yeast [Hayles et al. 1986a,b; Brizuela et al. 1987; Hindley et al. 1987], budding yeast [Hadwiger et al. 1989; Tang and Reed 1993], and humans [Richardson et al. 1990], the Xe-p9 protein is most similar [91% identical] to the human Cks2 homolog [Fig. 1B].

**Characterization of Xe-p9 in Xenopus egg extracts**

Because the physiological functions of the Sucl/Cks proteins have not been elucidated, we characterized the role of Xe-p9 in cell cycle progression in cell-free extracts from *Xenopus* eggs. As a first step toward this goal of studying the biochemistry of p9 in a completely homologous system, we prepared rabbit polyclonal antibodies against a peptide consisting of its 12 carboxy-terminal amino acids. These anti-p9 antibodies specifically recognized a single 9-kD polypeptide present in extracts from unfertilized *Xenopus* eggs [Fig. 2A]. In quantitation experiments with known amounts of recombinant p9, we determined that the endogenous concentration of p9 in egg extracts is ~5 ng/µl. Significantly, this concentration is similar to that of the Cdc2 protein kinase, a likely physiological partner of p9. In other immunoblotting experiments, we determined that the abundance of p9 is approximately constant through early *Xenopus* embryogenesis until at least stage 26 [data not shown]. Therefore, p9 appears to be a participant in both the embryonic and somatic cell cycles during early *Xenopus* development.

Next, we examined the behavior of p9 throughout an in vitro cell cycle in *Xenopus* egg extracts. We immunoblotted samples from the egg extracts at various points through the cell cycle with anti-p9 antibodies. Neither the abundance nor the electrophoretic mobility of p9 varied during the progression from interphase to mitosis [Fig. 2B]. In parallel, we determined by immunoprecipitation with anti-p9 antibodies that p9 can be found in a complex with Cdc2 and cyclin B2 in M-phase extracts from unactivated eggs [Fig. 2C, lanes b–d]. The association of p9 with Cdc2 was also detected in interphase extracts lacking cyclin B2 because of treatment with cycloheximide [Fig. 2C, lanes e–g]. In other experiments, we found that p9 also can associate with Cdk2 [data not shown]. Collectively, these experiments establish that p9 is a subunit of the Cdc2/cyclin B complex and perhaps of other Cdns present in *Xenopus* egg extracts.

**Excess Xe-p9 delays mitosis in Xenopus egg extracts**

As one approach to examine the function of Xe-p9, we
Xe-p9, a Xenopus homolog of Suc1

increased its overall concentration in Xenopus egg extracts by adding exogenous p9 and assessed the effect on cell cycle progression. For this purpose, we first expressed p9 as a 6-histidine fusion protein (HisGp9) in baculovirus-infected Sf9 insect cells (Fig. 2A). We observed that HisG-p9 could elicit a readily observable delay of mitosis after addition to Xenopus egg extracts, as judged by the time at which nuclei in the extracts underwent nuclear envelope breakdown (NEB; Fig. 3A). Typically, control egg extracts enter M phase (mitosis) ~90 min after activation. At a final concentration of 10 ng/μl, exogenously added p9 elicited a mitotic delay of ~1 hr, whereas at higher concentrations (25–50 ng/μl), NEB occurred ~2 hr later than in control extracts. At 10 ng/μl of His6–p9, the delay in NEB was mirrored by a corresponding lag in the activation of Cdc2-associated H1 kinase activity (Fig. 3B). Intriguingly, at higher concentrations of p9 (e.g., 50 ng/μl), Cdc2-associated H1 kinase activity (Fig. 3B) never rose above 20% of the peak level in control extracts, even though nuclear disassembly eventually did occur in these extracts.

To characterize the p9-dependent inhibition of mitosis in greater detail, we examined the phosphorylation state of Cdc25, the Cdc2-specific tyrosine phosphatase, at various times in the presence and absence of exogenously added His6–p9 (Fig. 3C). Normally, Cdc25 becomes activated at mitosis because of extensive phosphorylation that results in an electrophoretic mobility shift from 70 to 98 kD [Izumi et al. 1992, Kumagai and Dunphy 1992]. As expected, in a control extract lacking His6–p9, the Cdc25 protein underwent phosphorylation upon entry into mitosis (90 min after activation of the extract) (Fig. 3C, lanes a–f). In contrast, in an extract containing 10 ng/μl of His6–p9, we observed that the phosphorylation of Cdc25 was delayed significantly (Fig. 3C, lanes g–l). In particular, the complete activation of Cdc25 occurred at 165 min, which coincided with the time of mitosis in this His6–p9-treated extract. Finally, at a concentration of 50 ng/μl of His6–p9, we found that activation of Cdc25 did not occur even after ~5 hr (Fig. 3C, lanes m–q), despite the fact that the nuclei in these extracts had broken down by this time. However, upon immunoblotting of these extracts with an anti-Xenopus lamin L,, antibody, we found that the nuclear breakdown that occurred at late times in extracts containing 50 ng/μl of His6–p9 was accompanied by substantial proteolysis of the lamin L,, protein (data not shown). Therefore, in these particular extracts, nuclear breakdown appears to resemble more closely an apoptotic process (Oberhammer et al. 1994) or aberrant nuclear disintegration, rather than a true mitotic event. Collectively, these findings indicate that exogenously added His6–p9 can cause a substantial or essentially complete inhibition of mitotic entry depending on the final concentration of this protein.

Xe–p9 cannot delay mitotic induction by the Cdc2–AF mutant

In principle, the p9-dependent inhibition of mitosis could occur at any one of numerous steps in the activation and/or action of the Cdc2 protein kinase. To pin-
tated to alanine and phenylalanine, respectively. We prepared a complex of Cdc2–AF and Acyclin B (a nondegradable mutant of human cyclin B1) and then added this Cdc2–AF/Acyclin B complex to cycloheximide-treated

**Figure 2.** Characterization of the Xe–p9 protein in Xenopus egg extracts. (A) Immunoblot of Xe–p9 in a CSF extract [2 μl] and purified recombinant His6–p9 from Sf9 cells using anti-p9 peptide antibodies. (B) The abundance and electrophoretic mobility of Xe–p9 is constant during the cell cycle. Aliquots [2 μl] from a CSF extract were removed at the indicated times after activation with CaCl₂ [0.4 mM] and processed for immunoblotting with anti-p9 peptide antibodies. The extract entered mitosis between 75 and 90 min. (C) Xe–p9 is associated with Cdc2 and cyclin B2. Xe–p9 was immunoprecipitated from cycloheximide-containing CSF extracts (M) or interphase extracts (I) with anti-p9 peptide antibodies. The antibodies were recovered by using protein-A-Sepharose beads [100 μl/ml]. After immunoprecipitation, the protein A beads were resuspended in a volume of HEPE5-buffered saline [pH 7.5] equal to that of the original extract. The protein A beads and supernatants [containing Cdc2 and cyclin B2 that had not bound to the antibodies] from the immunoprecipitation were then subjected to immunoblotting with antibodies against Xenopus Cdc2 and Xenopus cyclin B2. (Lane a) Initial CSF extract [2 μl]; (lanes b,e] 2 μl of supernatant from M-phase [CSF] and I-phase extracts, respectively; (lanes c,f) 2 μl of protein A bead fraction from M-phase and I-phase extracts, respectively; (lanes d,g) 10 μl of protein A bead fraction from M-phase and I-phase extracts, respectively.

Point more precisely the biochemical steps at which p9 delays mitosis in this system, we took advantage of a mutant of Cdc2, the Cdc2–AF mutant. This mutant cannot undergo inhibitory phosphorylation on either Thr-14 or Tyr-15 because these residues have been mu-
Xe-p9, a Xenopus homolog of Suc1

Figure 4. The delay of mitosis by Xe-p9 is dependent on phosphorylation of Cdc2 on Thr-14 and/or Tyr-15 and can be reversed by the Cdc2-AF mutant. (A) Egg extracts containing a mock-purified preparation from uninfected Sf9 cells [●] or 25 ng/μl His6-p9 [▲] added at 50 min postactivation were monitored for NEB (%) at 15-min intervals. (B) Cycloheximide [100 μg/ml] was added to the same extract used in A. At 50 min postactivation, when interphase nuclei were formed completely, the Cdc2-AF/Δcyclin B complex [8 nM] was added along with either 25 ng/μl of His6-p9 or a mock-purified preparation from uninfected Sf9 cells. At the indicated times, NEB (%) was determined.

extracts in the presence or absence of His6-p9 [Fig. 4]. As expected, the mutant Cdc2 complex induced mitosis efficiently in a control extract lacking His6-p9 [Fig. 4B], as judged by the time required for 100% NEB. In parallel, we examined the ability of this complex to trigger mitosis in an extract containing 25 ng/μl His6-p9. Significantly, there was no delay in the time course of mitosis elicited by the Cdc2-AF/Δcyclin B complex [Fig. 4B], even though this concentration of p9 caused a pronounced delay of mitosis (~120 min) in a control extract containing the endogenous wild-type Cdc2 and cyclin B proteins [Fig. 4A]. In similar experiments we observed that His6-p9 also caused a long delay of mitosis in extracts to which we had added only recombinant human Δcyclin B, which binds to the endogenous wild-type Cdc2 [data not shown]. These experiments indicate that the p9-dependent inhibition of mitosis operates directly or indirectly through phosphorylation of Cdc2 on Thr-14 and/or Tyr-15 residues.

Exogenously added His6-p9 hinders tyrosine dephosphorylation of the Cdc2 protein

In several experimental systems, the phosphatase inhib-
Patra and Dunphy

Kumagai and Dunphy (1992) and inactivation of the Wee1 kinase (Mueller et al. 1995a). As shown in Figure 5, we observed that the p9-elicted delay of mitosis was completely abrogated by the addition of 3 μM okadaic acid. Treatment with okadaic acid substantially accelerated the activation/phosphorylation of Cdc25 in extracts containing either 10 or 50 ng/μl of His6-p9 (Fig. 5A; see Fig. 3C for the effect of His6-p9 on the phosphorylation of Cdc25 in the absence of okadaic acid). Significantly, when we examined the tyrosine phosphorylation of Cdc2 in these extracts, we found that His6-p9 impaired the tyrosine dephosphorylation of Cdc2, even in the presence of okadaic acid (Fig. 5B). In the absence of His6-p9, complete dephosphorylation of Cdc2 and entry into mitosis took place concomitantly with the activation of Cdc25 (Fig. 5A,B). However, in the presence of His6-p9, a complete dephosphorylation of tyrosine on Cdc2 did not occur (Fig. 5B, lanes d–k), even though activation of Cdc25 took place within 15 min of the addition of okadaic acid. Apparently, once enough tyrosine-dephosphorylated Cdc2 has been generated, mitosis can occur, even if the dephosphorylation of Cdc2 has not proceeded to completion. Collectively, these observations suggest that His6-p9 interferes directly with the dephosphorylation of Cdc2 by the Cdc25 protein, even when it has acquired full phosphatase activity at mitosis.

To examine the effect of His6-p9 on the action of Cdc25 more directly, we prepared a recombinant complex of Cdc25/Xenopus cyclin B that had been phosphorylated in vitro on Tyr-15 by the Xenopus Wee1 tyrosine kinase in the presence of [γ-32P]ATP (Mueller et al. 1995a). After inactivation of the Xenopus Wee1, we added recombinant Xenopus Cdc25 in the absence and presence of His6-p9 (Fig. 5C). The Drosophila string protein was used as a positive control (Kumagai and Dunphy 1991). As expected, the Xenopus Cdc25 phosphatase could efficiently dephosphorylate the 32P-labeled Cdc2 protein in the absence of recombinant His6-p9. In contrast, in the presence of increasing concentrations of His6-p9, there was a marked decrease in the Cdc25-catalyzed tyrosine dephosphorylation of Cdc2. Taken together, these observations indicate that at concentrations higher than those normally present in a Xenopus egg extract, the recombinant p9 protein appears to inhibit the Cdc25-catalyzed tyrosine dephosphorylation of the Cdc2 protein kinase.

Xe-p9 is essential for the entry into mitosis

As a complementary approach to assess the physiological function of p9, we immunodepleted p9 from Xenopus egg extracts with anti-p9 antibodies and examined the consequences upon cell cycle progression. We first examined the contribution of Xe-p9 to the G2/M transition, as our data had indicated that addition of His6-p9 delays this transition. For this purpose, we treated interphase egg extracts with protein A–agarose beads containing either control antibodies or antibodies directed against the carboxy-terminal peptide of p9 (Fig. 6). After removal of the beads by centrifugation, we determined, by immunoblotting with anti-p9 peptide antibodies, that essentially all of the p9 protein (>99%) could be removed from the extracts by this procedure (Fig. 6A, lane c). Because egg extracts frequently contain two or more gene products for cell cycle control proteins (because of the polyploid nature of Xenopus), we also attempted to address the issue of whether the anti-p9 peptide antibodies are capable of recognizing various potential isoforms of p9. Therefore, we also immunoblotted the control and p9-depleted supernatants with antibodies that had been raised against the whole p9 protein. These antibodies also could not detect any p9 in the p9-depleted extract (Fig. 6A, lane f).

Next, we examined the capacity of the control and p9-depleted extracts to enter mitosis (Fig. 6B). Because the p9-depletion procedure consistently resulted in the removal of ~40% of the Cdc2 protein (data not shown), we decided to program the extracts to enter mitosis with a preparation of recombinant Cdc2/Xenopus cyclin B that contains equimolar amounts of each subunit (Kumagai and Dunphy 1995). This procedure ensured that both the control and p9-depleted extracts would contain equivalent concentrations of the Cdc2/Xenopus cyclin B complex. As expected, the control extract entered mitosis rapidly, as demonstrated by a rapid increase in H1 kinase activity (within 10 min) upon addition of the Cdc2/Xenopus cyclin B complex (Fig. 6B). In striking contrast, an equivalent concentration of Cdc2/Xenopus cyclin B in the p9-depleted extract was unable to trigger the entry into mitosis (Fig. 6B). Even after an incubation of 180 min, there was no increase in H1 kinase activity above baseline levels in the p9-depleted extract. To address the possibility that the immunodepletion of p9 might also have removed another protein that is essential for mitosis, we performed a rescue experiment by adding back recombinant His6-p9 (1 ng/μl, final concentration) to the p9-depleted extract. We observed that the capacity of the p9-depleted extract to enter mitosis was fully restored by the inclusion of recombinant His6-p9 (Fig. 6B). Collectively, these experiments indicate that p9 appears to be an essential component that is required for Xenopus egg extracts to enter mitosis normally.

In principle, the inability of p9-depleted interphase egg extracts to enter mitosis could result from a defect in the formation, activation, or action of the Cdc2/cyclin B complex. To evaluate these possibilities, we added cyclin B to p9-depleted extracts and monitored the phosphorylation content of Cdc2 as a function of time (Fig. 7A), which is an indicator of complex formation between Cdc2 and cyclin B. We observed that tyrosine phosphorylation of the endogenous Cdc2 that bound to cyclin B increased to high levels in the p9-depleted extracts (Fig. 7A, lanes i–j). Furthermore, the addition of exogenous His6-p9 restored the ability of the p9-depleted extract to dephosphorylate the tyrosine residue of Cdc2 (Fig. 7A, lanes k–o). These observations indicate that in the absence of p9, Cdc2 can bind efficiently to cyclin B and also can undergo tyrosine phosphorylation, but the subsequent tyrosine dephosphorylation of Cdc2 cannot take place normally.
To analyze the defect in the p9-depleted extracts further, we also added the mutant Cdc2-AF\(\Delta\)cyclin B complex, which cannot undergo the inhibitory tyrosine and threonine phosphorylations, to control and p9-depleted interphase extracts. Significantly, we observed that the Cdc2-AF\(\Delta\)cyclin B complex could induce mitosis equally well in the control and p9-depleted extracts (Fig. 7B). Similar results were obtained when we added a Cdc2-AF\(\Delta\)cyclin B complex in which the Thr-161 residue had not been phosphorylated in vitro in S99 cell lysates [Kumagai and Dunphy 1995] before addition to the p9-depleted egg extracts (data not shown). These observations indicate that in p9-depleted extracts the Cdc2/cyclin B complex can undergo the activating Thr-161 phosphorylation normally. Moreover, the active complex is also able to phosphorylate many mitotic substrates in the absence of p9, as indicated by the occurrence of NEB. Therefore, the lack of p9 appears to cause a defect in the regulation of phosphorylation of Tyr-15 and/or Thr-14. In the absence of p9, Cdc2 could be either insensitive to the action of the Cdc25 phosphatase and/or possibly hypersensitive to the action of the Weel/Myt1 kinases. Alternatively, the defect might be attributed to an inability to activate Cdc25 and/or inhibit Weel/Myt1 at the G\(_2\)/M transition without the presence of p9 (see Discussion). In any case, removal of p9 from interphase egg extracts appears to impede the entry into mitosis as a consequence of a defect in the activation of the Cdc2/cyclin B complex.

**Xe-p9 is required for the destruction of cyclin B at mitosis**

In fission yeast, removal of the Suc1 protein, either by deleting the *suc1* gene or by down-regulating its expression with the thiamine-repressible *nm1* [no message in thiamine] promoter, leads to an arrest of the Suc1-deficient cells in mitosis with high levels of Cdc2-associated kinase activity [Moreno et al. 1989; Basi and Draetta 1995]. This arrest is accompanied by an accumulation of the Cdc13 mitotic cyclin protein [Basi and Draetta 1995], suggesting that the Suc1 protein might be required for the exit from mitosis. To assess the role of p9 in the metaphase-anaphase transition, we studied the ability of p9-depleted *Xenopus* egg extracts to degrade cyclin B at mitosis. In one approach, we added a complex of a full-length, degradable form of human cyclin B1 and the Cdc2-AF mutant to either control [mock-depleted] or p9-depleted interphase extracts (Fig. 8). We used the Cdc2-AF mutant because, as described above, the wild-type Cdc2/cyclin B complex cannot induce mitosis in a p9-depleted extract. As expected, the Cdc2-AF/cyclin B complex induced mitosis in both the control and p9-depleted extracts in ~45–60 min (Fig. 8). Furthermore, upon entry into mitosis, the human cyclin B protein was efficiently degraded in the control extract, as determined by immunoblotting with anti-cyclin antibodies (Fig. 8A, lanes a–g). In parallel, the Cdc2-associated H1 kinase activity in the control extract dropped precipitously (Fig. 8B). In strong contrast, however, the cyclin B protein in the p9-depleted extract remained stable for at least 90 min following entry into mitosis (Fig. 8A, lanes h–q) and the Cdc2-associated kinase activity remained high throughout this period (Fig. 8B). Finally, we added back recombinant His6-p9 [5 ng/\(\mu\)l] to the p9-depleted extract and found that both the destruction of cyclin B (Fig. 8A, lanes r–x) and the inactivation of Cdc2-associated kinase activity (Fig. 8B) occurred normally. Taken together, these observations indicate that p9 is essential for the ubiquitin-mediated proteolysis of cyclin B at mitosis.

To examine the role of p9 in cyclin destruction more fully, we also immunodepleted p9 from M-phase extracts from unfertilized *Xenopus* eggs (Fig. 9). In the absence of Ca\(^{2+}\), the cyclin B proteins in these extracts are stable because of the presence of the meiosis-specific cytostatic factor (CSF). The endogenous *Xenopus* cyclin B2 in control CSF extracts is degraded rapidly upon addition of Ca\(^{2+}\) (Fig. 9A). Likewise, the kinase activity of Cdc2 dropped rapidly in these control extracts (Fig. 9B). In striking contrast, the levels of both *Xenopus* cyclin B2 (Fig. 9A) and Cdc2-associated kinase activity (Fig. 9B) remained high for at least 3 hr after the addition of Ca\(^{2+}\) to extracts lacking p9. Importantly, this defect in cyclin degradation and Cdc2 inactivation also could be restored by the addition of recombinant His6-p9 [5 ng/\(\mu\)l]. Therefore, the destruction of cyclin B requires the presence of p9 in both meiotic and mitotic extracts.

**Discussion**

In this study we have isolated and characterized a *Xenopus* homolog (Xe-p9) of the small Cdk-associated subunit that was originally named Suc1 in fission yeast [Hayles et al. 1986a,b; Brizuela et al. 1987]. This protein appears to be a ubiquitous subunit of Cdks in all eukaryotes. The fission yeast Suc1 protein and its budding yeast homolog Cks1 are both essential for cell viability [Hindley et al. 1987; Hadwiger et al. 1989]. In human cells, there are at least two Suc1/Cks1 homologs (Ckshs1 and Ckshs2) that appear to be expressed differentially during the cell cycle [Richardson et al. 1990]. The importance of the Suc1/Cks1 protein in cell cycle control is underscored by the fact that Xe-p9 is essential for cell cycle progression in *Xenopus* egg extracts. Because these extracts recapitulate the simple embryonic cell cycle, our findings indicate that the Suc1/Cks1 protein is an integral component of the basic machinery that regulates Cdk function.

Despite its essential nature, the exact physiological function of the Suc1/Cks1 protein has remained mysterious. Although this protein is indispensable for cell division in both fission and budding yeast, overexpression of Suc1/Cks1 in yeast paradoxically leads to a mitotic delay [Hayles et al. 1986b]. Similarly, in biochemical experiments with heterologous components, the recombinant fission yeast Suc1 protein was found to inhibit the activation of the Cdc2/cyclin B complex in *Xenopus* oocyte cytosol fractions by blocking the tyrosine dephosphorylation of Cdc2 [Dunphy and Newport 1989]. In other studies the removal of Suc1/Cks1 function in bud-
Figure 6. Interphase extracts lacking p9 cannot enter mitosis. (A) Xe-p9 can be removed quantitatively from Xenopus egg extracts with anti-p9 peptide antibodies. Cycloheximide-treated interphase egg extracts (10 μl) were immunodepleted with protein A beads containing either 10 μg of anti-p9 peptide antibodies [lanes c,f] or 10 μg of control rabbit anti-mouse antibodies [mock; lanes b,e]. Aliquots (2 μl) from initial interphase extracts [lanes a,d] or from the antibody-treated supernatants were processed for immunoblotting with anti-p9 peptide antibodies [lanes a-c] or anti-p9 whole protein antibodies [lanes d-f]. (B) Cell cycle progression in p9-depleted extracts. Demembranated sperm nuclei (500 μl) were added to mock [●] and p9-depleted [■] extracts shown in A. In addition, 1 ng/μl of His6-p9 was added to an aliquot of the p9-depleted extract [▲]. After a 40-min incubation since addition of sperm chromatin, when membrane assembly around the chromatin was complete, a recombinant Cdc2/Δcyclin B complex [15 nm] was added. Subsequently, Cdc2-associated H1 kinase activity [in arbitrary units] was determined at 10-min intervals.

Figure 7. Xe-p9 controls the activation of MPF through the regulation of Tyr-15 and/or Thr-14 residues. (A) Tyrosine-phosphorylated Cdc2 accumulates in p9-depleted interphase extracts. Demembranated sperm nuclei were added to a mock-depleted extract, a p9-depleted extract, and a p9-depleted extract containing 4 ng/μl of His6-p9. After a 40-min incubation, histidine-tagged Δcyclin B [40 nm] was added. At the indicated times after addition of cyclin, Δcyclin B/Cdc2 complex was isolated with Ni-IDA-Sepharose beads and the phosphotyrosine content of Cdc2 was determined by immunoblotting with anti-phosphotyrosine antibodies. The time of mitosis (100% NEB) is denoted with a circle [note that the p9-depleted extract did not enter mitosis]. (B) Mitosis can occur in p9-depleted egg extracts containing a Cdc2 mutant that cannot be phosphorylated on Thr-14 and Tyr-15. Demembranated sperm nuclei were added to mock-depleted (●) or p9-depleted (▲) extracts. After a 40-min incubation, the Cdc2-APiAcyclin B complex (8 nm) was added, and Cdc2-associated H1 kinase activity [in arbitrary units] was determined at 15-min intervals.
Collectively, our studies indicate that p9 has multiple roles in regulating the biochemistry of the Cdc2/cyclin B complex.

By adding His6–p9 to interphase egg extracts and removing the endogenous p9 protein from these extracts by immunodepletion, we have found that p9 has a pronounced effect on the tyrosine phosphorylation of Cdc2 during the progression from interphase to mitosis. Overexpression of p9 in the egg extracts by the addition of recombinant His6–p9 leads to an arrest of the extracts in interphase. This effect appears to be attributable to an inhibition of the tyrosine dephosphorylation of Cdc2 because [1] the extracts treated with His6–p9 accumulate tyrosine-phosphorylated Cdc2 to high levels, and [2] His6–p9 blocks the tyrosine dephosphorylation of a recombinant Cdc2/cyclin B complex by the purified Cdc25 protein. Significantly, the removal of p9 from interphase extracts by immunodepletion with anti-p9 antibodies also abolishes entry into mitosis. In the p9-depleted extracts, tyrosine-phosphorylated Cdc2 likewise accumulates to high levels. This observation suggests that in the absence of p9, Cdc25 cannot dephosphorylate Cdc2 and/or the Wee1 and Myt1 kinases phosphorylate Cdc2 more efficiently. Our studies appear to favor the former possibility in that the initial rate of tyrosine phosphorylation of Cdc2 is comparable in extracts containing or lacking p9. At first glance, it would appear paradoxical that high concentrations of recombinant p9 inhibit the Cdc25-mediated tyrosine dephosphorylation of Cdc2, whereas the removal of p9 from egg extracts also leads to a defect in the tyrosine dephosphorylation of Cdc2. A simple expla-
nation would be that p9 facilitates the interaction between the Cdc2/cyclin B complex and the Cdc25 protein but that supraphysiological concentrations of p9 disrupt this process.

In another series of experiments, we found that immunodepletion of p9 from mitotic extracts also inhibits cell cycle progression. In particular, p9-depleted mitotic extracts cannot exit mitosis because of a defect in the degradation of cyclin B. In the absence of p9, the cyclin B protein in mitotic extracts remains stable, even though Cdc2 is fully active and would normally trigger proteolysis of cyclin B. The destruction of cyclin B in mitotic extracts from Xenopus eggs is carried out by the ubiquitin-dependent proteasomal pathway (Glotzer et al. 1991). This pathway includes three components (E1, E2, and E3) that are required for the ubiquitination of cyclin B (for a recent review, see Murray 1995). Subsequently, the ubiquitinated cyclin B molecules are degraded by the proteasome. The E3 component, also referred to as the anaphase-promoting complex or APC, is conserved in yeast and vertebrates (Imler et al. 1995; King et al. 1995; Tugendreich et al. 1995). Currently, the available evidence suggests that Cdc2 directly or indirectly activates the E3 component of the ubiquitin pathway at mitosis (Félix et al. 1990b; Luca et al. 1991; Hershko et al. 1994; King et al. 1995).

Our data indicate that the APC is unable to carry out the ubiquitination of a Cdc2/cyclin B complex in the absence of p9. It seems unlikely that the absence of p9 triggers the activation of the metaphase checkpoint, as this checkpoint is not operative in the Xenopus egg extracts under these experimental conditions (Minshull et al. 1994). Instead, the defect in cyclin degradation could result either from the inability of Cdc2 to activate the APC in the absence of p9 or the failure of the activated APC to recognize a Cdc2/cyclin B complex lacking p9. Superficially, our studies favor the latter possibility, because the active Cdc2–AF/cyclin B complex can induce mitosis efficiently in p9-depleted extracts. This observation implies that p9 is not absolutely required for Cdc2 to phosphorylate many of its mitotic substrates. However, a caveat to this view is that Cdc2 could require p9 for the recognition of a subset of crucial substrates such as the APC. Mechanistic studies with purified components will be required to resolve this issue definitively. However, it is striking that p9 also appears to be critical for the interaction of the Cdc2–AF/cyclin B complex with Cdc25, which is also phosphorylated on multiple sites by Cdc2 (Hoffmann et al. 1993; Izumi and Maller 1993). One plausible model is that p9 could act as a “docking factor” for Cdc2 regulators such as Cdc25 and the APC. This interaction could facilitate the phosphorylation of these regulators by Cdc2, but simultaneously it would enable the phosphorylated regulator to interact more efficiently with the Cdc2/cyclin B complex.

This model is borne out by recent crystallographic studies of the Suc1/Cks proteins from fission yeast (Bourne et al. 1995; Endicott et al. 1995; for review, see Endicott and Nurse 1995) and humans (Parge et al. 1993; Arvai et al. 1995), as well as the complex between human Cks1 and the human Cdk2 kinase (Bourne et al. 1996). Common features of the Suc1/Cks structures are a four-stranded β-sheet, a few α-helical regions, a patch of hydrophobic amino acids that could interact with Cdk5, and a cluster of basic residues that could bind a phosphate group. The interaction between Cks1 and the Cdk2 kinase positions this phosphate-binding site near the substrate recognition surface of Cdk2 so that it could have a role in the interaction of Cdk2 with its substrates (Bourne et al. 1996). We suggest that because two important regulators of the Cdc2/cyclin B complex, namely the Cdc25 phosphatase and the APC, are activated at mitosis by phosphorylation (Izumi et al. 1992; Kumagai and Dunphy 1992, Hershko et al. 1994; King et al. 1995), p9 could provide a suitable recognition surface for the binding of these regulatory molecules.

Another feature of Suc1/Cks structure is that these proteins may form dimers, at least under the conditions of crystallization (Parge et al. 1993; Bourne et al. 1995). It is not known whether the dimers of the Suc1/Cks proteins exist at the physiological concentrations found within eukaryotic cells. However, it is expected that the dimerization of these proteins would preclude association with Cdc2, because the dimer formation would reduce the accessibility of amino acid residues required for Cdk binding (Bourne et al. 1996). Therefore, the formation of oligomers in principle could represent a mechanism for negatively regulating Suc1/Cks1 function. Because p9 is critical for traversing both the G2/M and metaphase–anaphase transitions, oligomerization or some other structural change in p9 could have a pivotal role in regulating the passage through the checkpoints that govern these transitions.

A variety of genetic studies have been carried out on the role of the Suc1/Cks1 protein in the cell cycle of budding yeast and fission yeast. An important issue is whether the findings of these studies are consistent with our observations on p9 in Xenopus egg extracts. In fission yeast, the delay of mitosis that is observed upon overexpression of the Suc1 protein would be consistent with an inhibitory effect upon the tyrosine dephosphorylation of Cdc2. Likewise, the fact that overexpression of Xenopus p9 rescues the rad3-136 wee1-50 fission yeast mutant, which is deficient in the tyrosine phosphatase of Cdc2, could also be attributable to an inhibition of the dephosphorylation of Cdc2 on Tyr-15. Another type of genetic experiment on the role of the Suc1/Cks1 protein in fission yeast has involved the effect of its elimination on cell cycle progression (Moreno et al. 1989; Basi and Draetta 1995). Fission yeast with a disrupted suc1 gene entered mitosis but could not complete mitosis properly. The Cdc13 mitotic cyclin and its associated kinase activity accumulated to high levels in the mitotically arrested cells. Our finding that Xenopus egg extracts lacking p9 are defective in cyclin B degradation is entirely consistent with these genetic observations in fission yeast. Finally, fission yeast lacking the Suc1 protein can enter mitosis normally, whereas we find that p9-depleted Xenopus egg extracts cannot undergo the G2/M transition. The reason for this difference
is not known, but it may simply reflect a difference between these two species, as budding yeast with a defective Cks1 protein also cannot undergo the G2/M transition [Tang and Reed 1993].

In conclusion, we have demonstrated that p9 is an essential component of the Cdc2/cyclin B complex in Xenopus egg extracts. In these studies we have observed that during interphase, p9 has a role in regulating the phosphorylation of Cdc2 at Tyr-15. Furthermore, at the end of mitosis, p9 is essential for the recognition of the cyclin B subunit of MPF by the ubiquitin-dependent proteolysis machinery. Ultimately, a detailed structural analysis of the p9/Cdc2/cyclin B complex and its regulators (e.g., the Cdc25 phosphatase, the Wee1 and Myt1 kinases, and the enzymes of the ubiquitin-mediated degradation pathway) may provide a complete molecular view of Suc1/Cks1 function.

Materials and methods

Yeast transformation

The S. pombe strain rad3-136 wee1-50 ura4-D18 leu1-32 h- (Seaton et al. 1992) was transformed with a Xenopus oocyte cDNA library [Mueller et al. 1995a] using the spheroplast transformation procedure [Allshire 1990; Moreno et al. 1991]. In this library the cDNAs were directionally ligated into the cloning vector pAX-NMT, so that the 5' and 3' ends of the cDNAs are flanked by Apal and Xhol restriction sites, respectively. The expression of the cDNAs is under the control of the nmt1 promoter. Transformants were selected at 35.5°C on PM (minimal medium) plates containing 1.2 M sorbitol and 2% glucose, but lacking leucine. Potential rescuants were streaked again on PM plates lacking leucine followed by an additional round of selection at 35.5°C to eliminate false positives. Plasmids were recovered from these rescued strains and retransformed into the same mutant strain to confirm their rescuing capacity. One class of rescuing cDNA clones was found to have very high homology to the human p9\textsuperscript{Xe} protein [Richardson et al. 1990] and was selected for further study. The GenBank accession number is U56426.

Preparation of histidine-tagged p9 in Sf9 insect cells

To prepare a full-length recombinant p9 containing a 6-histidine tag at its amino-terminal end (His\textsuperscript{6}–p9), the initiating codon [ATG] in the cDNA clone was converted into an Ndel restriction site by PCR, where the 5' primer was 5'-GGTCCACATAT-GTCAATAAGAGACACTTAC-3' and the 3' primer was 5'-ACATCTAGAAGTTCATTTCTGTTGATCTTTTGG-3'. The PCR (in a final volume of 100 \mu l) contained 100 \mu g of the Xe–p9 cDNA clone and 2.5 units of Taq polymerase (Perkin Elmer, Norwalk, CT) in the buffer recommended by the manufacturer. The following PCR program was used. The reaction mixture was heated at 94°C for 2.5 min followed by 95°C for 0.5 min. It was then cycled five times at 94°C for 1 min, 47°C for 2 min, and 72°C for 3 min. This was followed by 20 cycles at 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min. Finally, the PCR product was extended at 72°C for 5 min. The PCR product was directly subcloned into pCR II vector using the TA cloning kit (Invitrogen, San Diego, CA) and sequenced. The Ndel–Xbal fragment was then cloned into the Ndel–Xbal site of pVL1393N–HIS [Tang et al. 1993]. The resulting vector was used for the production of His\textsuperscript{6}–p9 in Sf9 insect cells [Kumagai and Dunphy 1995; Mueller et al. 1995a].

Antibody production

Rabbits were immunized with a carboxy-terminal peptide of p9 (LLFRPPLKDDQK) that had been conjugated to keyhole limpet hemocyanin through the use of a cysteine that had been added to the amino-terminus of the peptide. Anti-Xe–p9 peptide antibodies were purified by affinity chromatography on an Affi-Gel 10 column (Bio-Rad, Richmond, CA) containing covalently bound peptides [Kumagai and Dunphy 1992]. Antibodies to the whole p9 protein were obtained by immunizing rabbits with the purified His\textsuperscript{6}–p9 protein. These antibodies were then affinity purified in a similar manner on a CNBr-activated Sepharose 4B column (Pharmacia, Piscataway, NJ) containing His\textsuperscript{6}–p9. Antibodies against phosphoerythrose (4G10) and the human cyclin B1 were obtained from Upstate Biotechnology (Lake Placid, NY).

Immunodepletion of p9 from CSF and interphase egg extracts

Typically, CSF and interphase egg extracts were immunodepleted with anti-Xe–p9 peptide antibodies and protein A–Sepharose beads (Sigma Chemical, St. Louis, MO). For this purpose, protein A–Sepharose beads (0.1 volume of the egg extract) were incubated with the antibody (91 \mu g/ml of egg extract) under constant agitation at 4°C for 1 hr. Subsequently, the beads were washed twice with HEPES-buffered saline (10 mM HEPES [pH 7.5], 150 mM NaCl) followed by two washes with XB buffer [Murray 1991]. CSF or interphase egg extracts containing cycloheximide (100 \mu g/ml) were then added to these washed beads and incubated at 4°C for 1 hr under constant agitation. Following this incubation, immunodepleted extracts were obtained by separating the protein A–Sepharose beads by centrifugation.

Assay of Cdc25-mediated tyrosine dephosphorylation of Cdc2

Cdc2 radiolabeled on Tyr-15 was prepared by treating a recombinant Cdc2/cyclin B complex with the Xenopus Wee1 tyrosine kinase in the presence of \([\gamma-32P]ATP\) as described [Mueller et al. 1995a]. Following the phosphorylation reaction, the Xenopus Wee1 kinase was inactivated by the addition of 20 mM EDTA (pH 8.0) and incubation at 37°C for 20 min. The radiolabeled Cdc2/cyclin B complex was mixed with different amounts of purified His\textsuperscript{6}–p9 [as indicated in the figure legends] or with a mock-purified preparation from uninfected S9 insect cells and incubated on ice for 10 min in the presence of 20 mM Tris-HCl (pH 8.0), 1 mg/ml of ovalbumin, 10 mM EDTA, and 5 mM di-thiothreitol. Next, 6-histidine-tagged Xenopus Cdc25 (A. Kumagai and W. Dunphy, unpubl.) was added, and the samples were incubated at 25°C. Aliquots (20 \mu l) were removed at 10-min intervals, mixed with Laemmli’s gel sample buffer, and frozen immediately. The dephosphorylation of Cdc2 was determined by SDS-PAGE and autoradiography.

Miscellaneous methods

CSF extracts from unactivated Xenopus eggs were prepared as described [Murray 1991]. Activation of these egg extracts by the addition of CaCl\textsubscript{2} (0.4 mM) and visual monitoring of the assembly and disassembly of nuclei formed around sperm chromatin (500 demembranated Xenopus sperm nuclei per microliter of extract) were described previously [Kumagai and Dunphy 1995; Mueller et al. 1995a]. In some cases, cycloheximide (100 \mu g/ml) or okadaic acid (3 \mu M) was added as described in the figure legends. Histone H1 kinase assays were performed as described.
Patra and Dunphy

(1989). The Cdc2–AF/Acyclin B and the Cdc2–AF/cyclin B1 [full length] complexes were prepared by gel filtration chromatography using a SMART system (Pharmacia, Piscataway, NJ) or by a SMART system (Pharmacia, Piscataway, NJ). Immunoblotting with various antibodies was done as described (Coleman et al. 1993) using 125I-labeled protein A (ICN, Cleveland, OH) or 125I-labeled sheep anti-mouse antibodies (Amersham, Arlington Heights, IL). Quantitation of kinase assays and immunoblots was performed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Typically, protein concentrations were determined using a Bio-Rad protein assay kit with bovine serum albumin or lysozyme as the standard. Coimmunoprecipitation of Xenopus Cdc2 and cyclin B2 with Xe-p9 in egg extracts was performed using 91 μg/ml of anti-Xe-p9 peptide antibodies as described (Mueller et al. 1995a).

Acknowledgments

We thank the members of the Dunphy laboratory for comments on the manuscript. We are grateful to G. Krohne (German Cancer Research Center, Heidelberg, Germany) for mouse monoclonal antibodies against Xenopus lamin [L], and to S. Subramani (University of California, San Diego) for the S. pombe rad3-136 wee1-50 strain. D.P. thanks P. Mueller for the Xenopus oocyte cDNA library and Akiko Kumagai for the antibodies against the Xenopus Cdc25, Xenopus Cdc2, Xenopus cyclin B2, and for providing the baculovirus constructs for histidine-tagged human cyclin B1 and histidine-tagged Xenopus Cdc25. D.P. was supported by a fellowship from the Leukemia Society of America. W.G.D. is an investigator of the Howard Hughes Medical Institute.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 USC section 1734 solely to indicate this fact.

References


Brizuela, L., G. Draetta, and D. Beach. 1987. p13<sup>sucl</sup> acts in the fission yeast cell division cycle as a component of the p34<sup>cdc2</sup> protein kinase. EMBO J. 6: 3507–3514.


Hayles, J., S. Aves, and P. Nurse. 1986b. sucl+ is an essential gene involved in both the cell cycle and growth in fission yeast. EMBO J. 5: 3373–3379.


Hindley, J., G. Phear, M. Stein, and D. Beach. 1987. Sucl+ encodes a predicted 13-kilodalton protein that is essential for cell viability and is directly involved in the division cycle of
Xe-p9, a Xenopus Suc1/Cks homolog, has multiple essential roles in cell cycle control.

D Patra and W G Dunphy


References

This article cites 69 articles, 22 of which can be accessed free at: http://genesdev.cshlp.org/content/10/12/1503.full.html#ref-list-1

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or click here.