Phosphorylation by Cdc28 Activates the Cdc20-dependent Activity of the Anaphase-promoting Complex

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Abstract. Budding yeast initiates anaphase by activating the Cdc20-dependent anaphase-promoting complex (APC). The mitotic activity of Cdc28 (Cdk1) is required to activate this form of the APC, and mutants that are impaired in mitotic Cdc28 function have difficulty leaving mitosis. This defect can be explained by a defect in APC phosphorylation, which depends on mitotic Cdc28 activity in vivo and can be catalyzed by purified Cdc28 in vitro. Mutating putative Cdc28 phosphorylation sites in three components of the APC, Cdc16, Cdc23, and Cdc27, makes the APC resistant to phosphorylation both in vivo and in vitro. The nonphosphorylatable APC has normal activity in G1, but its mitotic, Cdc20-dependent activity is compromised. These results show that Cdc28 activates the APC in budding yeast to trigger anaphase. Previous reports have shown that the budding yeast Cdc5 homologue, Plk, can also phosphorylate and activate the APC in vitro. We show that, like cdc28 mutants, cdc5 mutants affect APC phosphorylation in vivo. However, although Cdc5 can phosphorylate Cdc16 and Cdc27 in vitro, this in vitro phosphorylation does not occur on in vivo sites of phosphorylation.

Key words: mitosis • budding yeast • Cdc5 • Cks1 • Pds1

Introduction

Proteolysis plays a critical role in the eukaryotic cell cycle. During the exit from mitosis, ubiquitin mediated proteolysis destroys an inhibitor of sister chromatid separation (Pds1 in budding yeast and Cut2 in fission yeast; Holloway et al., 1993; Funabiki et al., 1996; Yamamoto et al., 1996) and the mitotic cyclins (Clb1–Clb4 in budding yeast; Ghiara et al., 1991; Glotzer et al., 1991; Yamano et al., 1996). These proteins are targeted for degradation by the anaphase-promoting complex (APC) or cyclosome, which is the E3 ubiquitin ligase for cyclins (King et al., 1995; Sudakin et al., 1995; Zecherlie et al., 1996; Pds1 (Cohen-Fix et al., 1996; Funabiki et al., 1997), and other substrates (Juang et al., 1997; Prinz et al., 1998; Shirayama et al., 1998), marking them for destruction by the 26S proteasome. The APC is regulated by the binding of two conserved activators, Cdc20 and Hct1 (also known as Cdh1; Schwab et al., 1997; Visintin et al., 1997; Faig et al., 1998; Kitamura et al., 1998; Lorca et al., 1998). In budding yeast, Cdc20-dependent APC activity initiates the metaphase to anaphase transition and the series of events that activate the Hct1-dependent APC, which induces complete mitotic cyclin destruction (Lim and Surana, 1996; Visintin et al., 1997; Shirayama et al., 1999). Hct1 acts in conjunction with the cyclin-dependent kinase (Cdk) inhibitor Sic1 to induce the rapid drop in Cdc28-associated kinase activity that drives cells out of mitosis and into the next G1 (Madden, 1993; Donovan et al., 1994; Amon, 1997; Li and Cai, 1997). The Hct1- and the Cdc20-dependent APC can both target Pds1 for destruction (Visintin et al., 1997; Rudner et al., 2000), suggesting that the main difference between them is the time during the cell cycle when each is active (Prinz et al., 1998).

Phosphorylation of Hct1 by Cdc28/Clib complexes keeps it from binding or activating the APC (Zecherlie et al., 1996; Jaspersen et al., 1999). This phosphorylation is removed by Cdc14, a phosphatase that is activated after Cdc20-dependent destruction of Pds1, Clb2, and the S-phase cyclin, Clb5 (Visintin et al., 1998; Jaspersen et al., 1999; Shirayama et al., 1999; Yeong et al., 2000). The late activation of Cdc14 ensures that cells do not inactivate Cdc28 and exit mitosis until well after they have initiated sister chromatid segregation.

Cdc20 is regulated in at least three ways: the gene is transcribed only in mitosis, the protein is targeted for destruction by the APC, and Cdc20 activity is inhibited by the spindle checkpoint, which monitors whether chromo-
somes have attached to the spindle properly (Weinstein, 1997; Fang et al., 1998a; Hwang et al., 1998; Kallio et al., 1998; Kimm et al., 1998; Kramer et al., 1998; Prinz et al., 1998; Shirayama et al., 1998).

The Cdc20-dependent A PC is regulated by phosphorylation. A PC subunits are phosphorylated in fission yeast, frogs, clams, and mammalian tissue culture cells (Hershko et al., 1994; Peters et al., 1996; Yamauchi et al., 1997; K. Otani et al., 1998). Phosphorylation correlates with A PC activity in vivo, and experiments in vitro have suggested that phosphorylating the A PC regulates Cdc20 binding and A PC activity (K. Otani et al., 1998, 1999; Shteingber et al., 1999). Studies in frog egg extracts and mammalian tissue culture cells have shown that the protein kinase Plk (known as Cdc5 in budding yeast and Plx1 in frogs) and the complex of Cdc2, Cyclin B, and Cks1, a small Cdk binding protein, can phosphorylate the A PC in vitro. Depletion of either Cks1 or Plx1 from frog extracts blocks cyclin destruction, suggesting that both Cdc2 and Plx1 may activate the A PC (Patra and Dunphy, 1996; Descombes and Nigg, 1998; K. Otani et al., 1998), but the relative importance of these two kinases in vivo is unclear. Phosphorylation of the A PC by a MAP-dependent protein kinase A inhibits the A PC both in vivo and in vitro (Yamauchi et al., 1996; K. Otani et al., 1998). Lastly, protein phosphatase 2A (PP2A) inhibits the A PC (Lahav-Baratz et al., 1995; Shteingber et al., 1999), whereas PP1 activates the A PC (Yamauchi et al., 1997).

In the accompanying paper (Rudner et al., 2000), we show that CDC28-T18V, Y19F (CDC28-VF), and other mutants with altered mitotic Cdc28 activity are compromised in activating the Cdc20-dependent A PC, revealing a requirement for Cdc20 in A PC activation. Here, we show that CDC28-VF is defective in the mitotic phosphorylation of the A PC and that this phosphorylation depends on Cdc20 activity both in vivo and in vitro. M utating potential phosphorylation sites in the A PC components Cdc16, Cdc23, and Cdc27 reduces Cdc20 binding to the A PC and Cdc20-dependent A PC activity in vivo.

Materials and Methods

Strain and Plasmid Construction

Table I lists the strains used in this work. A II strains are derivatives of the W303 strain background (W303-3a; R. Rothstein, Columbia University, New York, N Y). Standard genetic techniques were used to manipulate yeast strains (Sherman et al., 1974) and standard protocols were used for DNA manipulation (Maniatis et al., 1982). All deletions and replacements were confirmed by PCR or by mutant phenotype. The sequences of all primers used in this study are available upon request. The bacterial strains 2a and D Hs were used for amplification of DNA.

BAR1 was deleted using pG GS1 (a gift of J. Thorner, University of California, Berkeley, CA). CDC27-MBP strains were made by crossing K1890 (a gift of Mike Mendenhall, University of Kentucky, Lexington, KY); 12CA5 ascites (B. Crouch); rabbit polyclonal anti-Cdc16, anti-Cdc26 antibody (2006); 12CA5 ascites (B. Crouch); rabbit polyclonal anti-Cdc16, anti-Cdc26, and anti-Cdc27 (Lamb et al., 1994); and rabbit polyclonal anti-Cdc26 antibody (Hwang and Murray, 1995), except that the blocking reagent used was 2% BSA, PBS. Short spindles are bipolar spindles <2 μm long.

Immunoprecipitation and Western Blots

Immunoprecipitation, Western blots, A PC assay, and Cdc20 binding to the A PC were performed as described in the accompanying paper (Rudner et al., 2000). Modifications of the basic protocol are detailed below.

To resolve the phosphorylated forms of Cdc27 by Western blot, samples were electrophoresed on a 12.5% polyacrylamide gel containing 0.25% bisacrylamide. The phosphorylated forms of Cdc27 were resolved by Western blot on a 10% polyacrylamide gel containing 0.1% bisacrylamide.

The following antibodies were used in this study: 9E10 ascites (BabCO); affinity-purified rabbit polyclonal anti-Cbl2 and anti-Cib3 antibodies (K. Elayog and Murray, 1995); rabbit polyclonal anti-Sic1 serum (a gift of Mike Mendenhall, University of Kentucky, Lexington, KY); 12CA5 ascites (B. Crouch); rabbit polyclonal anti-Cdc16, anti-Cdc23, and anti-Cdc27 (Lamb et al., 1994); and rabbit polyclonal anti-Cdc26 antibody (Hwang and Murray, 1995). Details on the use of these antibodies can be found in the accompanying paper (Rudner et al., 2000).

In Vivo Labeling of the A PC

Yeast cells were arrested in G1 with alpha factor, in S-phase with HU, and in mitosis by spindle checkpoint activation and temperature shift. Once the cells were arrested at the indicated stage of the cell cycle, 50 ml of OD600 0.8 cells were harvested by centrifugation, washed twice in H2O, and resuspended in 1 ml phosphate-free complete synthetic medium (Rothblatt and Scheikman, 1989) containing 0.5-1 mcI [32P]PO4 (A mer sham Pharmacology, Biotech). Cells were labeled for 1 h, harvested by centrifugation, washed once in H2O, and then frozen in screw-cap tubes (Sarstedt). These tubes were used throughout the procedure to prevent radioactive contamination. The frozen yeast pellets were processed for immunoprecipitation as described in the accompanying paper (Rudner et al., 2000).
with the following modifications. 2–3 μg anti-Cdc26 antibody was bound to 20 μl protein A beads for 20 min on ice. These beads were then incubated with 10–20 μg of unlabeled cell lysate made from cell 1–2 h. A 2 μg of Cdc5 antibody was bound to 40 μl protein A beads and incubated with rotation for 1–2 h. The beads were washed four times with kinase bead buffer (500 mM NaCl, 50 mM Tris-Cl, pH 7.4, 50 mM NaF, 5 mM EDTA, 0.3% Triton X-100; transferring the beads to fresh tubes after the fourth wash), and then twice with 50 mM Tris-Cl, pH 7.5. The beads were then rotated in 50 mM Tris-Cl, pH 7.5, containing 0.5 mg/ml RNAse A for 30 min at 4°C, washed an additional two times in kinase bead buffer (transferring the beads to fresh tubes after the second wash), and then a final wash in 50 mM Tris-Cl, pH 7.5.

**In Vitro Phosphorylation of the APC**

Cells were arrested in G1 by alpha factor, were harvested by centrifugation, frozen, and processed for immunoprecipitation. 10–15 mg of cell lysate was preclarified in 50 μl protein A beads, and then the APC was immunoprecipitated with 2 μg anti-Cdc26 antibodies that were prebound to protein A beads as described above. After immunoprecipitation, the beads were washed three times in kinase bead buffer (transferring the beads to fresh tubes after the second wash), and then twice in low salt kinase buffer (10 mM NaCl, 20 mM Hapes-KOH, pH 7.4, 5 mM MgCl2, 1 mM DTT), 5 ng of purified Cdc28-His6, 50 ng purified Cdc2-MBP (gifts of Jeff Ubersax, University of California, San Francisco, CA), and 100 ng purified Cks3 (see below) in 2 μl of kinase dilution buffer (300 mM NaCl, 25 mM Hapes-KOH, pH 7.4, 10% glycerol, 0.1 mg/ml BSA) were added to a 13 μl of low salt kinase buffer containing 10 μM okadaic acid (Calbiochem-Novabiochem). This reaction mix was added to the immunoprecipitated APC and incubated at 25°C for 20 min. The beads were then washed three times in kinase bead buffer containing 1 μM okadaic acid (transferring the beads to fresh tubes after the second wash), and then twice in low salt kinase buffer containing 1 μM okadaic acid. These washes remove CIB-2-MBP and proteolytic fragments of CIB-2-MBP, which are well phosphorylated and obscure APC phosphorylation. Cdc5 phosphorylation was performed by adding the following to immunoprecipitated APC in kinase reaction buffer (20 mM KCl, 20 mM Hapes-KOH, pH 7.4, 10% glycerol, 0.1 mg/ml BSA) added to 15 μl of Cdc5 kinase reaction buffer (20 mM KCl, 20 mM Hapes-KOH, pH 7.4, 2 mM MgCl2, 2 mM MnCl2), final concentrations in

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**Table I. Strain List**

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<th>Name</th>
<th>Relevant genotype*</th>
<th>Source</th>
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*All strains are isogenic to W303-1a (MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1).

1 All pGAL-MPS1 strains are derived from crosses with KH153.
factor-arrested cells (Hardy and Pautz, 1996; Charles et al., 1997) using lambda phosphatase (New England BioLabs, Inc.).

Results

Cdc28 Phosphorylates the APC In Vitro

M mutants that reduce mitotic Cdc28 activity have difficulty activating the Cdc20-dependent APC, suggesting that Cdc28 might phosphorylate the APC or Cdc20 (Rudner et al., 2000). Therefore, we asked if the budding yeast APC is phosphorylated in vitro. We used A PC that was isolated by immunoprecipitating cell lysates with antibodies against Cdc26, a nonessential component of the APC (H wang and M urray, 1997), and used these immunoprecipitates as a substrate for purified recombinant Cdc28/Cib2/Cks1 (a gift of J eff U bersax and D avid M organ, U niversity of C alifornia, S an F rancisco, C A ) in the presence of γ[32P]ATP. In A PC isolated from wild-type cells, three major bands and a single minor band were phosphorylated (Fig. 1, top). We determined the identity of these four bands by phosphorylating the A PC isolated from cells containing epitope-tagged subunits that change their molecular weight. If the band shifted up in the epitope-tagged A PC, we concluded that the phosphorylated protein is the A PC subunit. By this criterion, the protein at 97 kD is Cdc16, the protein at 85 kD is Cdc27, and the minor species at 65 kD is Cdc23 (Fig. 1, top). Similar experiments have shown the band at 42 kD is A cp9 (data not shown).

We do not think the phosphorylation of the A PC in these reactions is due to kinases that coimmunoprecipitate with the A PC; no labeling is seen in immunoprecipitates lacking added Cdc28/Cib2/Cks1. However, a kinase bound to the A PC might need to be activated by Cdc28, as has been reported for PIK phosphorylation of the mammalian A PC (K otani et al., 1998). Therefore, we tested whether Cdc5, the PIK homologue in budding yeast, was required for in vitro A PC phosphorylation (K itada et al., 1993). We isolated the A PC from a cdc5-1 mutant that had been arrested in G1 by alpha factor at 25°C and then shifted to the restrictive temperature of 37°C for one hour. This A PC is fully phosphorylated in vitro by Cdc28 (Fig. 1), showing that Cdc5 is not required for A PC phosphorylation in this in vitro assay. In addition, Cdc5 is not detectable in alpha factor-arrested cells (H ardy and P autz, 1996; Charles et al., 1998; Shi ra yama et al., 1998), or in anti-Cdc26 immunoprecipitates of the A PC, isolated from mitotic cells that contain Cdc5 (D avid M organ, personal communication; and data not shown).

The APC Is Phosphorylated In Vivo

Is the A PC phosphorylated in vivo? Wild-type cells were arrested at three points in the cell cycle: during G1 by adding alpha factor (a mating pheromone), during S-phase by adding HU (a DNA synthesis inhibitor), and in mitosis with nocodazole (a microtubule polymerization inhibitor). The arrested cells were labeled with [32P]P O 4 and the A PC was isolated by immunoprecipitating cell lysates with antibodies against Cdc26. Three major proteins of 97, 85, and 65 kD were strongly labeled in nocodazole-arrested cells, and to a lesser extent in HU- and alpha factor-arrested cells (Fig. 2 A). These three proteins do not precipitate from Cdc26A cells. The molecular weights of these proteins suggest that they are the A PC subunits Cdc16, Cdc27, and Cdc23, and mutating phosphorylation sites in these pro-
Figure 2. The APC is phosphorylated in vivo. A, APC phosphorylation is greatest in mitosis. Wild-type (ADR376) and cdc26Δ (LH307) were grown overnight in YEP + 2% glucose at 23°C to log phase and then arrested in G1 with alpha factor (1 μg/ml), in S-phase with hydroxyurea (200 mM), or in mitosis with nocodazole (10 μg/ml) for 3 h. Cells were then transferred to phosphate-free CSM + 2% glucose containing 32PO₄, and alpha factor, HU, or nocodazole as indicated. After 1 h cells were harvested, lysed, and the APC was immunoprecipitated with anti-Cdc26 antibody. Immunoprecipitates were run on a polyacrylamide gel that was subjected to either autoradiography (top) or Western blotting (bottom). B and C, CDC28-VF, clb2Δ, cdc28-1N, and cdc5-1 have reduced APC phosphorylation in vivo. All strains contain pGAL-MPS1. Wild-type (KH153), CDC28-VF (KH181), clb2Δ (ADR1606), cdc28-1N (ADR1899), cdc5-1 (JC165), and cdc26Δ (ADR2023) were grown overnight in YEP + 2% raffinose at 23°C to log phase, and were then transferred to YEP + 2% galactose for 4 h to arrest the cells in mitosis by Mps1 overexpression. Cells were then transferred to phosphate-free CSM + 2% galactose containing 32PO₄, and treated as described in A. In B, cells were arrested by Mps1 overexpression at 23°C, whereas in C cells were arrested at 35°C. In all experiments, the Western blots shown below the autoradiographs illustrate that the same amount of APC was immunoprecipitated in all strains (except for cdc26Δ strains, where no APC was precipitated).
teins abolishes in vivo phosphorylation of the APC (see below).

Since Cdc28/Cib complexes are inactive in G1, the differences in APC phosphorylation during different cell cycle stages suggests this reaction depends on Cdc28/Cib complexes. We tested this hypothesis directly by comparing the phosphorylation of the APC in CDC28-VF, clb2Δ, and cdc28-1N cells, three mutants that affect the mitotic activity of Cdc28 (Piggott et al., 1982; Surana et al., 1991; Grandin and Reed, 1993; Rudner et al., 2000). The cells were arrested in metaphase at 25°C (Fig. 2 B) or at 35°C (Fig. 2 C) by overexpressing Mps1 from the galactose inducible GAL1 promoter, which activates the spindle checkpoint. All three mutants reduce the phosphorylation of the APC by a factor of 2–4 compared with wild-type.

Previous studies have suggested that in mammalian tissue culture cells, the protein kinase Plk is primarily responsible for phosphorylating the APC (Kotani et al., 1998). A mutant in CDC5, the yeast homologue of Plk, cannot activate the Hct1-dependent APC (Charles et al., 1998). To determine whether the APC phosphorylation is dependent on Cdc5, we examined APC phosphorylation in a cdc5-1 mutant, arrested in metaphase by overexpressing Mps1 at a semirestrictive temperature of 35°C. We observed a similar reduction in APC phosphorylation as in CDC28-VF and cdc28-1N (Fig. 1 C), suggesting that Cdc5 contributes to APC phosphorylation in vivo.

To confirm the identities of the phosphorylated APC subunits and to determine if the APC is phosphorylated by Cdc28 in vivo, we mutated all the potential Cdc28 phosphorylation sites in Cdc16, Cdc23, and Cdc27. Using the weakest possible consensus phosphorylation site (serine or threonine, followed by proline; S/TP) as our criterion, we mutated six sites in Cdc16, one in Cdc23, and five in Cdc27. We refer to the resulting genes as CDC16-6A, CDC23-1A, and CDC27-5A. As Fig. 3 A shows, most of the mutated sites fit only the minimal S/TP motif and lack a nearby basic residue found in many biochemically determined Cdk phosphorylation sites (Brown et al., 1999).

We directly assessed the ability of the mutant subunits to be phosphorylated in vivo and in vitro. In vivo, each alanine-substituted subunit is resistant to phosphorylation (Fig. 3 B). This result confirms our conclusion that Cdc16, Cdc27, and Cdc23 are the three major phosphorylated

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**Figure 3.** The APC is phosphorylated on potential Cdc28 phosphorylation sites. A, All serine/proline (SP) and threonine/proline (TP) sites on Cdc16, Cdc23, and Cdc27 were mutated to alanine/proline (AP). B, Phosphorylation site mutants are resistant to phosphorylation in vivo. All strains contain pGAL-MPS1. Wild-type (KH153), CDC16-6A (ADR1975), CDC23-A-1A (ADR1973), CDC27-5A-HA (ADR1974); and CDC16-6A CDC23-A CDC27-5A (ADR1979) and cdc26Δ (ADR2023) were grown in the presence of 32PO4 as described in Fig. 1 B. C, Phosphorylation site mutants are resistant to phosphorylation in vitro. The APC was isolated and phosphorylated in vitro as described in Fig. 1 for: cdc26Δ (LH307), CDC23-A (ADR2030), wild-type (ADR376), CDC27-5A (ADR2031), CDC16-6A (ADR2029), and CDC16-6A CDC23-A CDC27-5A (ADR2032).
proteins in the APC (Fig. 2) and shows that among the phosphorylation sites we mutated are the relevant in vivo sites. In addition, the phosphorylation of the different subunits are largely independent of each other. For example, the CDC23-A mutant eliminates the phosphorylation of Cdc23, but not that of Cdc16 and Cdc27. In vitro, Cdc23-A and Cdc27-5A are resistant to phosphorylation in vitro by Cdc28 (Fig. 3 C). Cdc16-6A is still weakly phosphorylated, though much less than the wild-type protein.

The APC Binds to Cks1
During the course of this work we discovered that the budding yeast APC, like the animal APC, can bind to Cks1-coupled beads (Sudakin et al., 1997). This interaction is thought to be critical for APC phosphorylation and reflects the ability of Cks1 to bring Cdc2/Cyclin B complexes into proximity with the APC by interacting with both complexes simultaneously (Patra and Dunphy, 1998; Shtein-
Cdc27 is critical for Cks1 binding to the APC. Reduced recovery of the APC does not reflect decreased binding of Cdc28-VF to Cks1 beads, since equal amounts of Cdc28-VF/Clb2 and Cdc28/Clb2 are recovered with the beads.

Fig. 4 A also shows that Cdc27 runs as a doublet on Western blots, with the upper band predominating in wild-type and the lower band predominating in Cdc28-VF. The slower mobility form of Cdc27 is a phosphorylated form because it can be converted to the faster one by treating the Cks1-bound material with lambda phosphatase (Fig. 4 B). We are also able to detect phosphorylation-dependent mobility shifts in Cdc16 and Cdc23 (data not shown). These phosphorylation-dependent shifts confirm our in vivo labeling data (Fig. 2) that show the APC is phosphorylated in vivo.

To investigate the relationship between reduced Cks1 binding and reduced Cdc27 phosphorylation in CD C28-VF cells, we followed both through the cell cycle. Fig. 4 C shows that through most of the cell cycle Cdc27 is partially phosphorylated, but as cells go through mitosis and Clb2 levels peak, Cdc27 and Cdc16 phosphorylation increases (Fig. 4 C, arrow). The amount of phosphorylation on both subunits increases further when nocodazole treatment arrests cells in mitosis by activating the spindle checkpoint. In CD C28-VF cells, as Clb2 levels increase, Cdc27 phosphorylation decreases before eventually returning to its partially phosphorylated state (Fig. 4 C, bracket).

These changes in APC phosphorylation correlate with its ability to bind Cks1-coupled beads (Fig. 4 C, bottom). In wild-type, no APC binds Cks1-coupled beads in an alpha factor arrest, and its binding increases as levels of Clb2 rise. In CD C28-VF cells, little binding of the APC to Cks1-coupled beads is seen at any stage of the cell cycle, even though the peak levels of Clb2 are similar in wild-type and CD C28-VF cells. These differences suggest that mitotic phosphorylation by Cdc28 is required for APC binding to Cks1-coupled beads (Sudak et al., 1997). A through the difference in APC phosphorylation between wild-type and CD C28-VF cells in a synchronous cell cycle is transient and subtle, it is reproducible, and it correlates with a large difference in the ability of the APC to bind Cks1-coupled beads.

We next tested whether APC phosphorylation is required for the APC to bind Cks1-coupled beads. The beads do not bind an APC containing Cdc27-5A (Fig. 4 D), but do bind an APC containing either Cdc16-6A and Cdc23-A. This result suggests that phosphorylation of Cdc27 is critical for Cks1 binding to the APC.

We have shown that mutations that alter the mitotic activity of Cdc28 have reduced APC phosphorylation (Fig. 2, B and C). Our ability to detect APC phosphorylation on Western blots allowed us to examine additional mutants that affect Cdc28 activity. Mutants that reduce the mitotic activity of Cdc28 (CD C28-VF, cd28-1N, clb2D, and cks1-38) are hypersensitive to checkpoint arrest caused by overexpression of Mps1, whereas a mutant that primarily affects G1 activity (cd28-84) is not (Reed, 1980; Surana et al., 1991; Tang and Reed, 1993; Rudner et al., 2000). To test if this correlation extended to the phosphorylation state of the APC, we arrested these strains in mitosis with nocodazole and immunoblotted for Cdc27 and Cdc16. This analysis correlates perfectly with our earlier findings: cdc28-4 have normal levels of Cdc16 and Cdc27 phosphorylation, whereas clb2D, cd28-1N, and cks1-38 all have reduced levels and resemble CD C28-VF (Fig. 4 E).

**APC Phosphorylation Site Mutants Affect Mitotic, but Not G1 Functions**

We wanted to rule out the possibility that the phosphorylation site mutants had general effects on the activity of the APC, as opposed to a specific effect on its mitotic, Cdc20-dependent form. Since the Hct1-dependent APC is maximally active when Cdc28 is inactive, loss of Cdc28-dependent phosphorylations should not affect Hct1-dependent APC activity in G1-arrested cells that lack active Cdc28 (Zachariae et al., 1998; Jaspersen et al., 1999).

Therefore, we examined the activity of APC containing the alanine-substituted subunits that had been isolated from G1-arrested cells. A PC activity was measured in an in vitro ubiquitination assay that uses an iodinated fragment of sea urchin cyclin B as a substrate and the APC provided from anti-Cdc26 immunoprecipitates (Charles et al., 1998). Fig. 5 shows that there is no difference in G1-specific APC activity between wild-type cells and those carrying alanine mutations in APC subunits (CD C16-6A, CD C23-A, or CD C27-5A). Thus, the mutations in putative Cdc28-dependent phosphorylation sites have not disrupted the ability of these subunits to associate with other APC components or produce normal levels of Hct1-dependent
A PC activity. In addition, cells carrying alanine-substituted A PC subunits show no obvious growth defects at any temperature.

We asked if the alanine substitutions in the A PC, like CDC28-VF, have difficulty leaving mitosis (Rudner et al., 2000). Wild-type and CDC16-6A CDC23-A CDC27-5A cells were arrested in G1 by the mating pheromone alpha factor and then released into the cell cycle. Once cells had budded, alpha factor was readded to arrest cells that had completed the cycle. CDC16-6A CDC23-A CDC27-5A cells show a 20-min delay in sister chromatid separation (Fig. 6 A). Cib2 and Cib3 proteolysis are delayed by >40 min. This defect is not due to slower mitotic entry, because wild-type and CDC16-6A CDC23-A CDC27-5A cells initiate budding, degrade Sic1, and form a short mitotic spindle at the same time (Fig. 6 A and data not shown).

Mutating A PC phosphorylation sites also causes an increased sensitivity to spindle checkpoint arrest caused by Mps1 overexpression (Hاردwick et al., 1996; Rudner et al., 2000). Serial dilutions of wild-type, CDC28-VF, mutants in single A PC subunits, double mutants, and the triple mutant were spotted on plates where Mps1 is induced to high levels (Fig. 6 B). Both CDC16-6A and CDC27-5A are sensitive to Mps1 overexpression and combining the two mutate.
The alanine-substituted APC is defective in Cdc20-dependent APC function. A, Pds1 is stabilized in anaphase in CDC16-6A and CDC27-5A. cdc15-2 GAL-PDS1-HA (ADR 1968), cdc15-2 CDC27-5A GAL-PDS1-HA (ADR 1999), and cdc15-2 CDC16-6A GAL-PDS1-HA (ADR 2003) were grown overnight at 23°C in YEP + 2% raffinose to log phase and shifted to 37°C to arrest the cells in anaphase (raf). When >85% of the cells had reached anaphase (after 4 h, as judged by nuclear division, which was scored by 4',6-diamidino-2-phenylindole [DAPI] staining). Pds1-HA expression was induced for 1 h by the addition of 2% galactose, and at t = 0, its expression was terminated by the addition of 2% glucose. Samples were taken at the indicated times and processed for Western blots. Clb2 and Cdc27 are shown as a loading controls.

B, Cdc20 binding to the APC is impaired in CDC16-6A. cdc15-2 CDC20-myc12 (ADR 1979), cdc15-2 CDC27-5A CDC20-myc12 (ADR 1987), cdc15-2 CDC20-myc12 CDC16-6A (ADR 1990), and cdc26Δ CDC20-myc12 (ADR 2036) were grown overnight in YEP + 2% glucose at 23°C to log phase and transferred into fresh YEP + 2% glucose at 37°C. When >85% of the cells were arrested in anaphase (4 h, as judged by nuclear division, which was scored by DAPI staining), the cells were harvested, lysed, and the APC was immunoprecipitated with polyclonal anti-Cdc26 antibodies. The amount of Cdc20-myc12 bound to the APC was determined by Western blotting the immunoprecipitates with the 9E10 antibody. Equal amounts of Cdc23 were precipitated with the anti-Cdc26 antibodies (left) and equal amounts of cell lysate were used in the immunoprecipitation (right, cell lysate). cdc26Δ, which arrests in metaphase, not anaphase, accumulates high levels of Cdc20 because Cdc20 stability is regulated by the APC (Prinz et al., 1998; Shirayama et al., 1998).

The CDC23-A mutation alone has little phenotype, but exacerbates the effect of both the CDC16-6A and CDC27-5A mutations. These data suggest that phosphorylation of the APC subunits contribute to the ability to overcome the spindle checkpoint and suggest that the alanine-substituted APC, like CDC28-VF, may be defective in the Cdc20-dependent APC.

To test Cdc20-dependent APC function more directly, we examined the ability of these non-phosphorylatable APC mutants to support Pds1 degradation in vivo. Pds1 is normally unstable in anaphase with a half-life of about ten minutes (Jaspersen et al., 1998). We arrested wild-type and non-phosphorylatable APC mutants in anaphase (using the cdc15-2 mutant), induced Pds1 expression from the G AL1 promoter by adding galactose for one hour, and then shut the promoter off by adding glucose and examined the rate of Pds1 degradation. Previously, we have shown that in this anaphase arrest, CDC28-VF and clb2Δ stabilize Pds1 (Rudner et al., 2000). We see a similar effect when the CDC27-5A and CDC16-6A mutants are combined with cdc15-2. The half-life of Pds1 is increased to 30 min in anaphase-arrested CDC27-5A, and to >90 min in CDC16-6A cells (Fig. 7A).

We also have examined the association of Cdc20 with the APC in the alanine-substituted mutants at the cdc15-2 block, a time when the Cdc20-dependent APC is active. This association is impaired in CDC28-VF (Rudner et
We arrested cdc15-2, cdc15-2 CDC16-6A, and cdc15-2 CDC27-5A cells in anaphase, immunoprecipitated the A PC with anti-Cdc26 antibodies, and examined the amount of associated Cdc20. The association of Cdc20 to the A PC is reduced in CDC27-5A cells and severely impaired in CDC16-6A cells (Fig. 7 B).

The Role of Cdc5 in APC Phosphorylation

Different groups debate whether Cdc2 (Cdk1) or Plk is the major A PC kinase in vivo (Lahav-Baratz et al., 1995; Kotani et al., 1998; Patra and Dunphy, 1998; Shiebeyn and Hershko, 1999; Shiebeyn et al., 1999). Since we find that A PC phosphorylation is reduced in a cdc5-1 mutant (Fig. 2 C), we tested whether purified recombinant Cdc5 could phosphorylate the A PC in vitro. Like Cdc28, Cdc5 phosphorylates Cdc16, Cdc27, and A pc9 (Fig. 8 A), but unlike Cdc28, appears to not phosphorylate Cdc23. Cdc5 also phosphorylates proteins that have the molecular weights of several other A PC subunits (A pc1, -2, -4, and -5; Zachariae et al., 1996). We have not confirmed the identity of these proteins because there is little evidence that these proteins are major targets of phosphorylation in vivo (Fig. 2). The ability of human Plk to activate the A PC in vitro depends on pretreatment with Cdc2/Cyclin B complexes (Kotani et al., 1998), a result that has been interpreted to suggest that Cdc2 activates Plk’s kinase activity against the A PC. In our hands, however, Cdc5’s ability to phosphorylate the A PC does not increase when the kinase is pretreated with purified Cdc28/C1b2/Cks1 complexes (data not shown).

We next tested whether purified Cdc5 can phosphorylate the alanine-substituted A PC. The Cdk sites we mutated on Cdc16, Cdc23, and Cdc27 are also potential sites of phosphorylation by Cdc5. Substrates of the frog homologue of Cdc5, Plx1, become epitopes for the MPM-2 antibody after phosphorylation by Plx1 (Kumagai and Dunphy, 1996) and MPM-2 recognizes phosphorylation at SP or TP sites (Westendorf et al., 1993). In contrast to their effect on phosphorylation by Cdc28, the A PC phosphorylation site mutants had no effect on in vitro phosphorylation of the A PC by recombinant Cdc5 (Fig. 8 B). This observation makes it likely that the reduced in vivo A PC phosphorylation seen in cdc5-1 cells is an indirect effect of reduced Cdc5 activity, rather than a direct in vivo phosphorylation of these A PC subunits by Cdc5.

Discussion

We have shown that the budding yeast A PC subunits, Cdc16, Cdc23, and Cdc27, are phosphorylated in vivo and in vitro. Phosphorylation in vivo depends on Cdc28, and in vitro it is catalyzed by pure Cdc28/C1b2/Cks1 complexes. Mutating potential Cdc28 phosphorylation sites in Cdc16, Cdc23, and Cdc27 abolishes their in vivo phosphorylation and compromises the mitotic, but not the G1 functions of the A PC. We have also shown that Cdc5 affects A PC phosphorylation in vivo and can catalyze A PC phosphorylation in vitro. Our analysis of A PC phosphorylation site mutants in vivo and in vitro, however, argues that in vivo Cdc5 indirectly induces the phosphorylation of Cdc16,
Cdc23, or Cdc27, rather than directly modifying these subunits.

The APC Is Phosphorylated in Budding Yeast

Our results agree with studies on other organisms that show mitosis-specific APC phosphorylation. Cdc16, Cdc23, Cdc27, and Apc1 are phosphorylated in frogs; Apc1, Cdc16, and Cdc27 are phosphorylated in mammalian tissue culture cells; and Cdc16 (Cut 9) is phosphorylated in fission yeast (Peters et al., 1996; Y amada et al., 1997; Patra and D unphy, 1998; K.otani et al., 1999). Although APC phosphorylation has been shown to activate the Cdc20-dependent APC in mammalian tissue culture and clam egg extracts (K.otani et al., 1998; Shteinberg et al., 1999), and Cks1 depletions prevent mitotic exit in frog extracts (Patra and Dunphy, 1996), this is the first report to examine the in vivo function of a Cdc28 phosphorylation. A thorough phosphorylation of Cdc16, Cdc23, and Cdc27 is not essential for viability in budding yeast, our studies suggest that it stimulates Cdc20-dependent APC activity and Cdc20 binding to the APC in vivo.

Cdc27 remains partially phosphorylated in G1 cells (Fig. 4 C). The presence of slower migrating Cdc27 in G1 cells could arise two ways: during the exit from mitosis, if Cdc28-catalyzed phosphorylation declines after phosphatases have been inactivated; or in G1, by phosphorylation catalyzed by another kinase. Because Cdc27-5A runs as a single band on Western blots in G1 (Fig. 5), we favor the possibility that G1 phosphorylation on Cdc27 remains from the previous mitosis. This finding would suggest that the phosphatase that removes phosphorylation from the APC is only active in mitosis. PP2A has been proposed to play such a role in clams and frogs (Lahav-Brarat et al., 1995; V orlaafer and Peters, 1998).

In one report, Pik has been identified as the major kinase of the mammalian APC (K.otani et al., 1998), although others have argued that this role is played by Cdc2 (Lahav-Brarat et al., 1995; Patra and Dunphy, 1998; Shteinberg and Hershko, 1999; Shteinberg et al., 1999). We asked if its budding yeast homologue, Cdc5, plays a similar role. In vivo, APC phosphorylation is reduced in the cdc5-1 mutant and purified Cdc5 phosphorylates the APC in vitro (Figs. 2 C and 8 A). Three observations argue that in living cells Cdc5 does not directly phosphorylate the APC subunits we have studied: phosphorylation site mutations that completely block phosphorylation of Cdc16, Cdc23, and Cdc27 in vivo, do not block in vitro phosphorylation of these subunits by Cdc5 (Fig. 8 B); purified Cdc28/CIb2/Cks1, that lacks detectable Cdc5, efficiently phosphorylates immunoprecipitated APC (Fig. 1); and the same mutations that block Cdc28-catalyzed phosphorylation in vitro also block in vivo APC phosphorylation (Fig. 3).

If Cdc5 does not phosphorylate Cdc16, Cdc23, and Cdc27 directly, how does it regulate the phosphorylation of these subunits? Cdc5 may be responsible for phosphorylating other APC subunits (Apc1, -2, -4, and -5 are potential substrates; Fig. 8) in vivo, and the phosphorylation of these subunits may affect the phosphorylation of the Cdc28 targets Cdc16, Cdc23, and Cdc27. Alternatively, Cdc5 may modulate Cdc28/CIb/Cks1 activity or localization.

Phosphorylation Stimulates Cdc20-dependent APC Activity

We have shown that phosphorylation site mutants in the APC reduce activation of the Cdc20-dependent APC. The half-life of Pds1 is increased in CD C27-5A and CD C16-6A cells, and this defect in Cdc20 function could be explained by the observed inability of Cdc20 to bind an APC containing Cdc16-6A. This data supports genetic experiments showing that reduced mitotic Cdc28 activity compromises the Cdc20-dependent APC (Rudner et al., 2000).

If Cdc20 binding and activity depend on a phosphorylated APC, why is the triple mutant CD C16-6A CD C23-A CD C27-5A viable? Even in the triple mutant there is some residual Cdc20 binding to the APC (data not shown), which is presumably sufficient to drive the metaphase to anaphase transition. The residual binding of Cdc20 to the APC could depend on the phosphorylation of other subunits. In support of this idea, we see weak phosphorylation of proteins we believe to be Apc1, -4, -5, and -9 in some in vivo labelings (data not shown), and a protein we believe to be Apc9 is phosphorylated in vitro by Cdc28/CIb2/Cks1 complexes (data not shown and Fig. 1; Zachariae et al., 1996). In addition, cdc28-1N, a mutation in Cdc28 that cannot bind Cks1 (Kaiser et al., 1999; and data not shown) and cks1-38, have reduced APC phosphorylation (Figs. 1 C and 4 E). These two mutants are temperature-sensitive for growth and arrest in mitosis (Piggott et al., 1982; Tang and Reed, 1993), suggesting that APC phosphorylation may be essential. Alternatively, it has been proposed that the primary defect in cdc28-1N and cks1-38 is in proteasome function (Kaiser et al., 1999), though proteasome activity was examined in G1, not in mitosis, leaving the relevance of this finding to the exit from mitosis uncertain.

Our data suggests that activation of the APC by phosphorylation opposes its inhibition by the spindle checkpoint. Although CD C16-6A CD C23-A CD C27-5A is viable, its delay in mitosis (Fig. 6 A) becomes lethal when the spindle checkpoint is activated (Fig. 6 B). Both APC phosphorylation and the spindle checkpoint affect the ability of Cdc20 to activate the APC, but have no effects on the G1, Hct1-dependent activity of the APC.

Regulation of APC Phosphorylation

Phosphorylation of the APC in frogs and clams in vitro depends on homologues of the small Cdk binding protein, Cks1, and in clams, Cks1 stimulates Cdc20-dependent APC activity (Patra and Dunphy, 1998; Shteinberg and Hershko, 1999; Shteinberg et al., 1999). In budding yeast, the role of Cks1 remains uncertain. Although we add purified Cks1 to our in vitro kinase reactions, Cks1 is not required for APC phosphorylation in vitro (Fig. 1 and data not shown). However, APC phosphorylation in vivo clearly depends on Cks1 (Fig. 4 E) and phosphorylation of Cdc27 is required for APC binding to Cks1-coupled beads (Fig. 4 D). We do not think that the binding of the APC to Cks1-coupled beads correlates with the ability of Cdc28 to phosphorylate the APC in vivo: although an APC containing Cdc27-5A does not bind to Cks1-coupled beads, Cdc16 and Cdc23 are fully phosphorylated in a CD C27-5A mutant. Despite this in vivo finding, we see reduced in
vitro phosphorylation of Cdc16 and Cdc23 in an A PC containing Cdc27-5A (Fig. 3 C).

Mutants that affect the mitotic form of Cdc28 have reduced levels of phosphorylation of the A PC, whereas cdc28-4 cells, which are defective in the G1 form of Cdc28 (Reed, 1980), show normal phosphorylation of Cdc27 and Cdc16. We were surprised to discover that A PC phosphorylation in cdc28-4 appears to be normal (Fig. 4 E), because this mutant has ~20% the amount of Cdc28 protein as wild-type cells at the permissive temperature of 23°C, and very little detectable Cdc28-associated kinase activity when immunoprecipitated from cell lysates (Surana et al., 1991; and data not shown). A possible explanation of the absence of mitotic defects in cdc28-4 cells is that the specific activity of each Cdc28-4 molecule is equal to that of wild-type Cdc28, although the total number of active kinases is drastically reduced. The specific activity of individual Cdc28 molecules may be critical for A PC phosphorylation because one Cdc28/Cbk2/Cks1 complex may bind persistently to the A PC. Once bound to the A PC, this single complex might be responsible for multiple phosphorylations. If the steady state phosphorylation of the A PC is determined by the balance between phosphorylation by Cdc28 and dephosphorylation by protein phosphatases, and Cdc28 remains bound to the A PC, a drop in specific activity of Cdc28 would reduce the phosphorylation and activity of the A PC.

How do cells escape from mitosis? If activating Cdc28/Cbk2/Cks1 complexes activates the Cdc20-dependent A PC, which in turn triggers chromosome segregation, how do cells ensure that the lag between activating Cdc28 and activating the Cdc20-dependent A PC is long enough to assemble a spindle and align chromosomes on it? A llthough one answer is that the spindle checkpoint inhibits Cdc20 in cells with misaligned chromosomes (Hwang et al., 1998; Kim et al., 1998), this explanation is not enough. Inactivating the spindle checkpoint does not kill yeast cells, implying other mechanisms exist to block premature activation of the Cdc20-dependent A PC. Another possible mechanism is regulating the abundance of Cdc20. High levels of CDC20 transcripts are restricted to mitotic cells and APC-dependent kinases. Cdc28 and dephosphorylation by protein phosphatases, and Cdc28 remains bound to the A PC, a drop in specific activity of Cdc28 would reduce the phosphorylation and activity of the A PC.

Rudner and Murray Cdc28 Phosphorylates the A PC

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Note Added in Proof. Similar results showing that Cdc20 only binds to a phosphorylated A PC have been published recently (Kramer, E. R., N. Scheuringer, V. Podrelojevik, M. Mann, and J. M. Peters. 2000. Mol. Biol. Cell. 11:1555–1569).

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