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)[1] or cyclosome, which is the E3 ubiquitin ligase for cyclins (King et al., 1995; Sudakin et al., 1995; Zachariae 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; Fang et al., 1998b; 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 (Mendenhall, 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/Clb complexes keeps it from binding or activating the APC (Zachariae et al., 1998; 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-

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[1]*Abbreviations used in this paper:* APC, anaphase-promoting complex; Cdk, cyclin-dependent kinase; HU, hydroxyurea; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A.

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somes have attached to the spindle properly (Weinstein, 1997; Fang et al., 1998a; Hwang et al., 1998; Kallio et al., 1998; Kim et al., 1998; Kramer et al., 1998; Prinz et al., 1998; Shirayama et al., 1998).

The Cdc20-dependent APC is regulated by phosphorylation. APC subunits are phosphorylated in fission yeast, frogs, clams, and mammalian tissue culture cells (Hershko et al., 1994; Peters et al., 1996; Yamada et al., 1997; Kotani et al., 1998). Phosphorylation correlates with APC activity in vivo, and experiments in vitro have suggested that phosphorylating the APC regulates Cdc20 binding and APC activity (Kotani et al., 1998, 1999; Shteinberg 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 APC in vitro. Depletion of either Cks1 or Plx1 from frog extracts blocks cyclin destruction, suggesting that both Cdc2 and Plx1 may activate the APC (Patra and Dunphy, 1996; Descombes and Nigg, 1998; Kotani et al., 1998, 1999), but the relative importance of these two kinases in vivo is unclear. Phosphorylation of the APC by cAMP-dependent protein kinase A inhibits the APC both in vivo and in vitro (Yamashita et al., 1996; Kotani et al., 1998). Lastly, protein phosphatase 2A (PP2A) inhibits the APC (Lahav-Baratz et al., 1995; Shteinberg et al., 1999), whereas PP1 activates the APC (Yamada 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 APC, revealing a requirement for Cdc28 in APC activation. Here, we show that *CDC28-VF* is defective in the mitotic phosphorylation of the APC and that this phosphorylation depends on Cdc28 activity both in vivo and in vitro. Mutating potential phosphorylation sites in the APC components Cdc16, Cdc23, and Cdc27 reduces Cdc20 binding to the APC and Cdc20-dependent APC activity in vivo.

Materials and Methods

Strain and Plasmid Construction

Table I lists the strains used in this work. All strains are derivatives of the W303 strain background (W303-1a; Rodney Rothstein, Columbia University, NY). 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 TG1 and DH5α were used for amplification of DNA.

BAR1 was deleted using pJGsst1 (a gift of Jeremy Thorner, University of California, Berkeley, CA). *CDC27-MBP* strains were made by crossing JC35 (a gift of Julia Charles, University of California, San Francisco, CA) to the appropriate strains. *cdc26*Δ strains were described previously (Hwang and Murray, 1997). *clb2*Δ strains were made by crossing K1890 (a gift of Kim Nasmyth, Institute of Molecular Pathology, Vienna, Austria) to the appropriate strains. *pCUP-GFP12-lacI12* and *lacO:LEU2* were integrated using pSB116 (Biggins et al., 1999) and pAFS59 (Straight et al., 1996), respectively. *pGAL-MPS1* strains were made with pAFS120 (Hardwick et al., 1996). *pGAL-PDS1-HA* strains were made by crossing RTK43 (a gift of Rachel Tinker-Kulberg, Johns Hopkins University, MD) to the appropriate strains. *APC9* was tagged by the PCR-targeting method. Cells were transformed with a cassette containing the bacterial KAN^R gene that confers G418 resistance in W303. The cassette was amplified by PCR from pFA6a-3HA-kanMX6 (Longtine et al., 1998) with primers containing the sequences that flank the stop codon of *APC9*. The construction of *CDC20-myc12* and *cks1-38* is described in Rudner et al. (2000, this issue).

Alanine-substituted mutants in *CDC16*, *CDC23*, and *CDC27* were made using site-directed mutagenesis (Kunkel, 1985). Mutations were confirmed by the introduction of new restriction enzyme sites and by sequencing (ABI). For *CDC16*, the EcoR1/Xho1 fragment of pWAM10 (Lamb et al., 1994) was cloned into KS (−) (Stratagene) to create pAR290. pAR290 was mutagenized to create pAR293, which contains all six serine/threonine to alanine substitutions. pAR294 was cut with EcoRI and NotI, and ligated to a EcoRI/PstI PCR fragment that contains the 3′ end of *CDC16*, a PstI/SpeI PCR fragment that contains the *TRP1* gene, and a SpeI/NotI PCR fragment that contains the 3′ untranslated region of the *CDC16* gene. The resultant plasmid, pAR303, was cut with XhoI and NotI, and integrated at the *CDC16* locus. The TRP^+ transformants were screened by PCR for the presence of all mutations. For *CDC23*, the BamHI/NotI fragment of pRS239 (Lamb et al., 1994) was cloned into KS (−) to create pAR228. pAR228 was mutagenized to create pAR240, which contains the single serine to alanine substitution in *CDC23*. pAR228 was cut with BamHI and NotI, transformed into *cdc23-1* cells (ADR1285), and selected for growth at 37°C. Transformants were screened by Western blot for the HA tag present at the 3′ end of the gene, and by PCR for the presence of the alanine substitution. For *CDC27*, the PstI/NotI fragment of pJL25 (Lamb et al., 1994) was cloned into KS (−) to create pAR201. pAR201 was mutagenized to create pAR203, which contains all five serine/threonine to alanine substitutions in *CDC27*. pAR203 was cut with NdeI and NotI, and ligated to a NdeI/XbaI PCR fragment that contains the KAN^R gene and a XbaI/NotI PCR fragment containing the 3′ untranslated region of *CDC27*. The resultant plasmid, pAR271, was cut with KpnI and NotI, and integrated at the *CDC27* locus. Transformants were screened by PCR for the presence of all mutations.

Physiology

Physiological experiments were performed as described in the accompanying paper (Rudner et al., 2000, this issue). Hydroxyurea (HU; Sigma-Aldrich) was added directly to media at a final concentration of 200 mM.

Cells were fixed for indirect immunofluorescence in 3.7% formaldehyde for 1 h. The spindles were visualized by antialpha-tubulin (Harlan Sera-Lab) immunofluorescence as described previously (Hardwick 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, APC assay, and Cdc20 binding to the APC 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 0.025% bisacrylamide. The phosphorylated forms of Cdc16 were resolved by Western blot on a 10% polyacrylamide gel containing 0.13% bisacrylamide.

The following antibodies were used in this study: 9E10 ascites (BabCO); affinity-purified rabbit polyclonal anti-Clb2 and anti-Clb3 antibodies (Kellogg and Murray, 1995); rabbit polyclonal anti-Sic1 serum (a gift of Mike Mendenhall, University of Kentucky, Lexington, KY); 12CA5 ascites (BabCO); rabbit polyclonal anti-Cdc16, anti-Cdc23, and anti-Cdc27 (Lamb et al., 1994); and rabbit polyclonal anti-Cdc26 antibody (Hwang and Murray, 1997). Details on the use of these antibodies can be found in the accompanying paper (Rudner et al., 2000).

In Vivo Labeling of the APC

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 OD_{600} 0.8 cells were harvested by centrifugation, washed twice in H_2O, and resuspended in 1 ml phosphate-free complete synthetic medium (Rothblatt and Schekman, 1989) containing 0.5–1 mCi $^{32}PO_4$ (Amersham Pharmacia Biotech). Cells were labeled for 1 h, harvested by centrifugation, washed once in H_2O, and were 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)

Table I. Strain List

Name	*MAT*	Relevant genotype*	Source
ADR313	**a**	*clb2Δ::LEU2*	This study
ADR376	**a**	*bar1Δ*	This study
ADR477	**a**	*CDC28-HA:URA3*	This study
ADR483	**a**	*cdc28-1N*	This study
ADR509	**a**	*CDC28-VF-HA:URA3*	This study
ADR842	**a**	*cdc28-4*	This study
ADR1252	**a**	*CDC28-VF-HA:URA3 bar1Δ*	This study
ADR1389	**a**	*CDC28-HA:URA3 bar1Δ*	This study
ADR1606	**a**	*clb2Δ:LEU2 ura3-1:pGAL-MPS1:URA3*	This study
ADR1705	**a**	*CDC27-MBP bar1Δ*	This study
ADR1767	**a**	*cks1Δ::KAN^R trp1-1:cks1-38:TRP1*	This study
ADR1790	**a**	*cdc15-2 CDC20-myc12 CDC28-HA:URA3*	This study
ADR1899	**a**	*cdc28-1N ura3-1:pGAL-MPS1:URA3*	This study
ADR1968	**a**	*CDC28-HA:URA3 trp1-1:pGAL-PDS1-HA:TRP1 bar1Δ*	This study
ADR1973	**a**	*CDC23-A-HA ura3-1:pGAL-MPS1:URA3*	This study
ADR1974	**a**	*CDC27-5A:KAN^R ura3-1:pGAL-MPS1:URA3*	This study
ADR1975	**a**	*CDC16-6A:TRP1 ura3-1:pGAL-MPS1:URA3*	This study
ADR1976	**a**	*CDC23-A-HA CDC27-5A:KAN^R ura3-1:pGAL-MPS1:URA3*	This study
ADR1977	**a**	*CDC16-6A:TRP1 CDC23-A-HA ura3-1:pGAL-MPS1:URA3*	This study
ADR1978	**a**	*CDC16-6A:TRP1 CDC27-5A:KAN^R ura3-1:pGAL-MPS1:URA3*	This study
ADR1979	**a**	*CDC16-6A:TRP1 CDC23-A-HA CDC27-5A:KAN^R ura3-1:pGAL-MPS1:URA3*	This study
ADR1987	**a**	*cdc15-2 CDC27-5A:KAN^R CDC20-myc12 CDC28-HA:URA3*	This study
ADR1990	**a**	*cdc15-2 CDC16-6A:TRP1 CDC20-myc12 CDC28-HA:URA3*	This study
ADR1999	**a**	*CDC27-5A:KAN^R trp1-1:pGAL-PDS1-HA:TRP1*	This study
ADR2003	α	*CDC16-6A:TRP1 trp1-1:pGAL-PDS1-HA:TRP1*	This study
ADR2023	**a**	*cdc26Δ::HIS3 ura3-1:pGAL-MPS1:URA3*	This study
ADR2029	**a**	*CDC16-6A:TRP1 bar1Δ*	This study
ADR2030	**a**	*CDC23-A-HA bar1Δ*	This study
ADR2031	**a**	*CDC27-5A:KAN^R bar1Δ*	This study
ADR2032	**a**	*CDC16-6A:TRP1 CDC23-A-HA CDC27-5A:KAN^R bar1Δ*	This study
ADR2036	α	*cdc26Δ::LEU2 CDC20-myc12*	This study
ADR2042	**a**	*APC9-HA3:KAN^R bar1Δ*	This study
ADR2061	**a**	*his3-11,15:pCUP1-GFP-lacI:HIS3 leu2-3,112:lacO:LEU2 bar1Δ*	This study
ADR2064	**a**	*CDC16-6A:TRP1 CDC23-A-HA CDC27-5A:KAN^R his3-11,15:pCUP1-GFP-lacI:HIS3 leu2-3,112:lacO:LEU2 bar1Δ*	This study
JC145	**a**	*cdc5-1 bar1Δ*	Julia Charles
JC165	**a**	*cdc5-1 ura3-1:pGAL-MPS1:URA3*	Julia Charles
K6180	**a**	*CDC16-myc6:URA3 balΔ*	Kim Nasmyth
KH153‡	**a**	*ura3-1:pGAL-MPS1:URA3*	Kevin Hardwick
KH181	**a**	*CDC28-VF-HA:URA3 ura3-1:pGAL-MPS1:URA3*	Kevin Hardwick
LH307	**a**	*cdc26Δ::LEU2 bar1Δ*	Lena Hwang
SLJ378	**a**	*CDC23-HA bar1Δ*	Sue Jaspersen

*All strains are isogenic to W303-1a (*MAT**a** ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*).
‡All *pGAL-MPS1* strains are derived from crosses with KH153.

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 mg of unlabeled cell lysate made from *cdc26Δ* cells for 1–2 h. After incubation, the beads were washed twice in lysis buffer. At the same time, the labeled cell lysate (typically 10 mg) was precleared in 75 μl protein A CL-4B Sepharose beads (Sigma-Aldrich) for 1 h, and then centrifuged at 14,000 rpm for 5 min at 4°C. The labeled lysate was then added to the antibody-bound 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 EGTA, 5 mM EDTA, 0.1% 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 precleared 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 Hepes-KOH, pH 7.4, 5 mM $MgCl_2$, 1 mM DTT). 5 ng of purified Cdc28-His_6, 50 ng purified Clb2-MBP (gifts of Jeff Ubersax, University of California, San Francisco, CA), and 100 ng purified Cks1 (see below) in 2 μl of kinase dilution buffer (300 mM NaCl, 25 mM Hepes-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 ATP, 2 μCi γ[^{32}P]ATP (Amersham Pharmacia Biotech), and 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 Clb2-MBP and proteolytic fragments of Clb2-MBP, which are well phosphorylated and obscure APC phosphorylation. Cdc5 phosphorylation was performed by adding the following to immunoprecipitated APC: purified His_6-HA-Cdc5 (a gift of Julia Charles, University of California, San Francisco, CA) in 5 μl of Cdc5 storage buffer (250 mM KCl, 20 mM Hepes-KOH, pH 7.4, 10% glycerol, 5 mM NaF, 0.1 mg/ml BSA) added to 15 μl of Cdc5 kinase reaction buffer (20 mM KCl, 20 mM Hepes-KOH, pH 7.4, 2 mM $MgCl_2$, 2 mM $MnCl_2$; final concentrations in

20 μl reaction) containing 10 μm ATP, 2 μCi γ[^{32}P]ATP, and 10 μm okadaic acid.

Cks1 Bead Pulldowns

Cks1 protein was made as described previously (Booher et al., 1993) using pCKS1-1. After the ammonium sulfate precipitation, the pellet was resuspended in lysis buffer (50 mM Tris-Cl, pH 8.0, 2 mM EDTA, 10% glycerol) and then desalted on a PD-10 column (Amersham Pharmacia Biotech) that had been equilibrated in CnBr coupling buffer (500 mM NaCl, 100 mM Na_2CO_3, pH 8.3). Cks1 was then coupled to CnBr-activated Sepharose 6MB or 4B (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Beads were washed and stored in lysis buffer (100 mM NaCl, 50 mM Tris-Cl, pH 7.5, 50 mM NaF, 50 mM Na-β-glycerophosphate, pH 7.4, 2 mM EGTA, 2 mM EDTA, 0.1% Triton X-100, 0.02% NaN_3). 3–5 mg of cell lysate was incubated with 10 μl Cks1-coupled beads for 1–2 h, washed three times in kinase bead buffer (500 mM NaCl, 50 mM Tris-Cl, pH 7.4, 50 mM NaF, 5 mM EGTA, 5 mM EDTA, 0.1% Triton X-100; transferring the beads to fresh tubes after the second wash), and then twice in low salt kinase buffer. Phosphatase treatment of Cks1 bead pulldowns was performed as previously described (Hardwick and Murray, 1995) using lambda phosphatase (New England Biolabs, Inc.).

Results

Cdc28 Phosphorylates the APC In Vitro

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 APC that was isolated by immunoprecipitating cell lysates with antibodies against Cdc26, a nonessential component of the APC (Hwang and Murray, 1997), and used these immunoprecipitates as a substrate for purified recombinant Cdc28/Clb2/Cks1 (a gift of Jeff Ubersax and David Morgan, University of California, San Francisco, CA) in the presence of γ[^{32}P]ATP. In APC 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 APC isolated from cells containing epitope-tagged subunits that change their molecular weight. If the band shifted up in the epitope-tagged APC, we concluded that the phosphorylated protein is the APC 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 Apc9 (data not shown).

We do not think the phosphorylation of the APC in these reactions is due to kinases that coimmunoprecipitate with the APC; no labeling is seen in immunoprecipitates lacking added Cdc28/Clb2/Cks1. However, a kinase bound to the APC might need to be activated by Cdc28, as has been reported for Plk phosphorylation of the mammalian APC (Kotani et al., 1998). Therefore, we tested whether Cdc5, the Plk homologue in budding yeast, was required for in vitro APC phosphorylation (Kitada et al., 1993). We isolated the APC 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 APC is fully phosphorylated in vitro by Cdc28 (Fig. 1), showing that Cdc5 is not required for APC phosphorylation in this in vitro assay. In addition, Cdc5 is not detectable in alpha factor-arrested cells (Hardy and Pautz, 1996; Charles et al., 1998; Shirayama et al., 1998), or in anti-Cdc26 immunoprecipitates of the APC, isolated from mitotic cells that contain Cdc5 (David Morgan, personal communication; and data not shown).

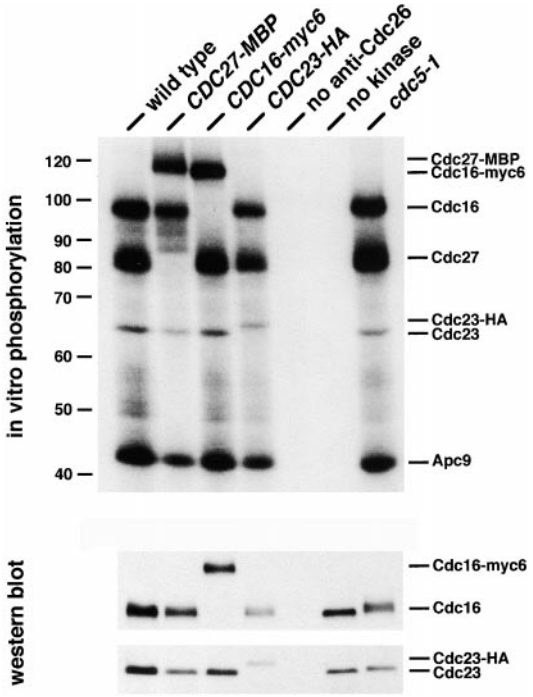


Figure 1. The APC is phosphorylated in vitro by Cdc28. Wild-type (ADR376), *CDC27-MBP* (ADR1705), *CDC16-myc6* (K6180), *CDC23-HA* (SLJ378), and *cdc5-1* (JC145) were grown overnight in YEP + 2% glucose at 23°C to log phase, arrested in G1 by alpha factor (1 μg/ml) for 3 h, harvested, lysed, and the APC immunoprecipitated with anti-Cdc26 antibody. The immunoprecipitates were treated with purified Cdc28-His_6, Clb2-MBP, Cks1, and γ[^{32}P]ATP, were washed to remove phosphorylated Clb2-MBP, and were then run on a polyacrylamide gel that was subjected to autoradiography (top) or Western blotting (bottom). *cdc5-1* cells were shifted to 37°C for an additional 1 h of alpha factor treatment. As controls, cell lysate was mock precipitated in the absence of anti-Cdc26 antibody (no anti-Cdc26) or was precipitated in the presence of anti-Cdc26 antibody, but no Cdc28, Clb2, or Cks1 was added to kinase reaction (no kinase). The Western blot shows that similar amounts of APC were precipitated with the anti-Cdc26 antibody.

The APC Is Phosphorylated In Vivo

Is the APC 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 $^{32}PO_4$ and the APC 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 *cdc26*Δ cells. The molecular weights of these proteins suggest that they are the APC subunits Cdc16, Cdc27, and Cdc23, and mutating phosphorylation sites in these pro-

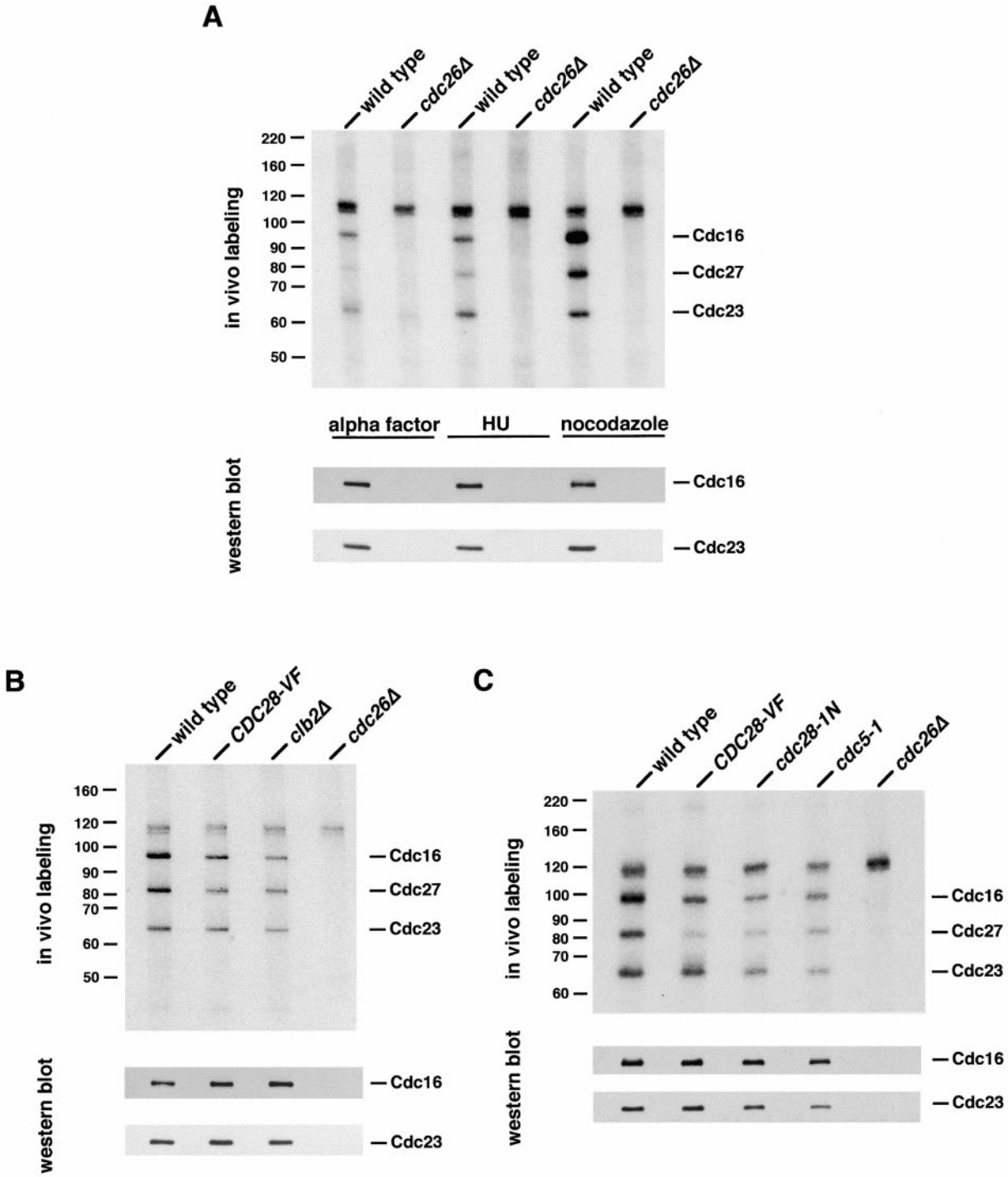


Figure 2. The APC is phosphorylated in vivo. A, APC phosphorylation is greatest in mitosis. Wild-type (ADR376) and *cdc26*Δ (LH307) were grown overnight in YEP + 2% glucose at 23°C to log phase and then arrested in G1 with alpha factor (1 μg/ml), in S-phase with hydroxyurea (200 mM), or in mitosis with nocodazole (10 μg/ml) for 3 h. Cells were then transferred to phosphate-free CSM + 2% glucose containing $^{32}PO_4$, 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*, *clb2*Δ, *cdc28-1N*, and *cdc5-1* have reduced APC phosphorylation in vivo. All strains contain *pGAL-MPS1*. Wild-type (KH153), *CDC28-VF* (KH181), *clb2*Δ (ADR1606), *cdc28-1N* (ADR1899), *cdc5-1* (JC165), and *cdc26*Δ (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 $^{32}PO_4$, 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 *cdc26*Δ strains, where no APC was precipitated).

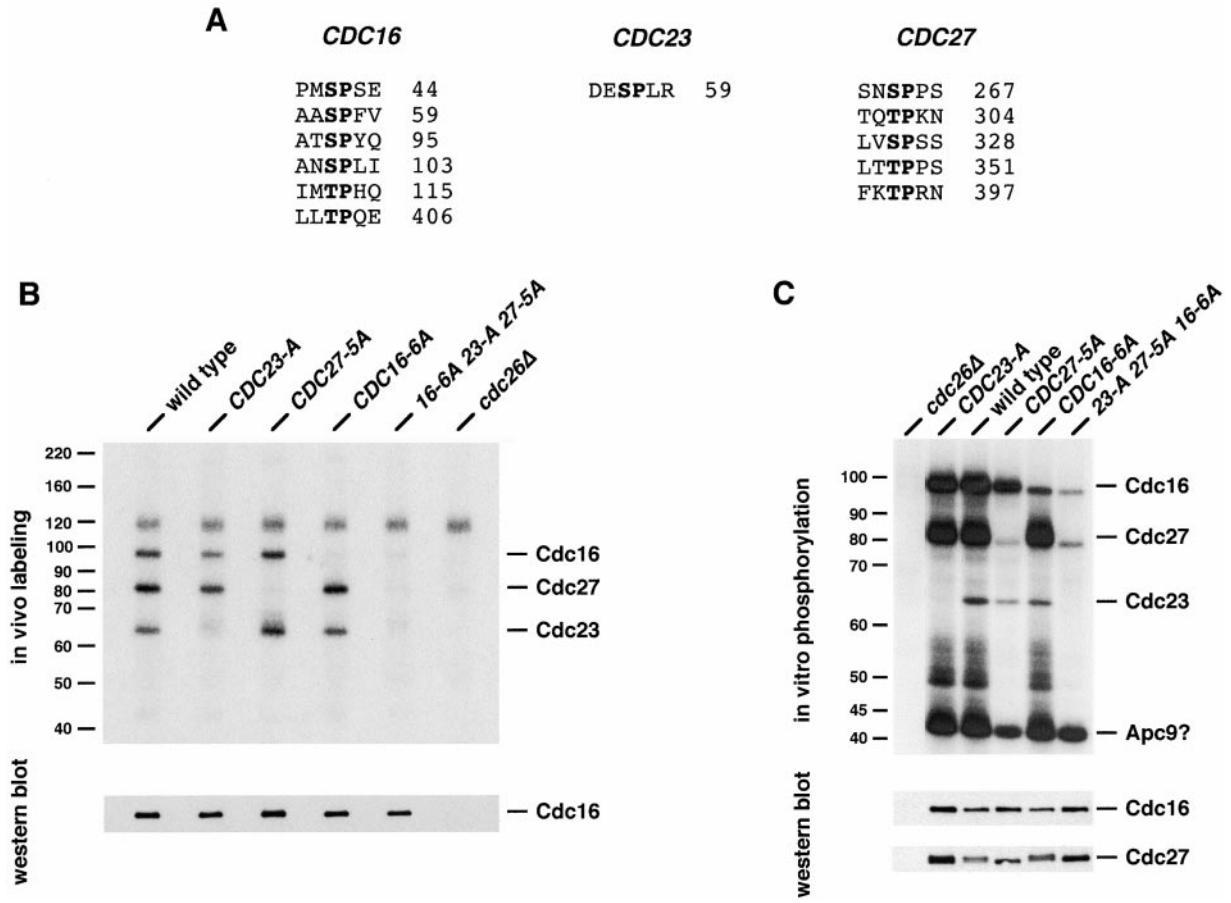


Figure 3. The APC is phosphorylated on potential Cdc28 phosphorylation sites. A, All serine/proline (SP) and threonine/proline (TP) sites on Cdc16, Cdc23, and Cdc27 were mutated to alanine/proline (AP). B, Phosphorylation site mutants are resistant to phosphorylation in vivo. All strains contain *pGAL-MPS1*. Wild-type (KH153), *CDC16-6A* (ADR1975), *CDC23-A-HA* (ADR1973), *CDC27-5A-HA* (ADR1974); and *CDC16-6A CDC23-A CDC27-5A* (ADR1979) and *cdc26*Δ (ADR2023) were grown in the presence of $^{32}PO_4$ 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: *cdc26*Δ (LH307), *CDC23-A* (ADR2030), wild-type (ADR376), *CDC27-5A* (ADR2031), *CDC16-6A* (ADR2029), and *CDC16-6A CDC23-A CDC27-5A* (ADR2032).

teins abolishes in vivo phosphorylation of the APC (see below).

Since Cdc28/Clb complexes are inactive in G1, the differences in APC phosphorylation during different cell cycle stages suggests this reaction depends on Cdc28/Clb complexes. We tested this hypothesis directly by comparing the phosphorylation of the APC in *CDC28-VF*, *clb2*Δ, 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 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 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-A* 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

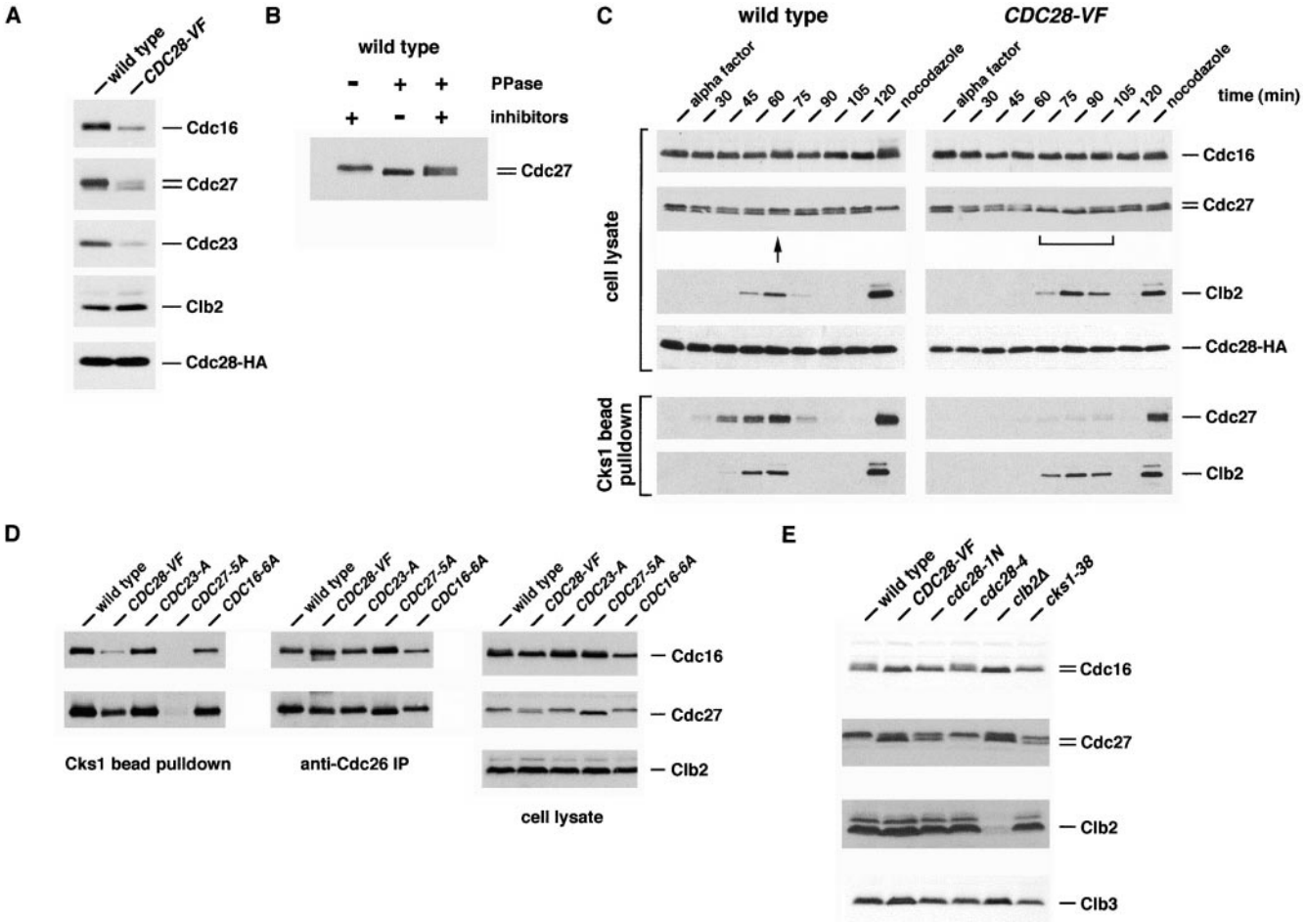


Figure 4. The APC associates with Cks1-coupled beads. A, The APC from *CDC28-VF* associates poorly with Cks-coupled beads. Wild-type (ADR477) and *CDC28-VF* (ADR509) were grown overnight in YEP + 2% glucose at 23°C to log phase and arrested in mitosis with nocodazole (10 μg/ml) for 3 h. Cells were harvested, lysed, and mixed with Cks1-coupled beads. Western blots of the material bound to the Cks1-coupled beads show that the APC and Cdc28/Clb2 bind to the beads. B, Cdc27 phosphorylation can be seen by Western blotting. Wild-type APC, isolated as described in A, was treated either with lambda phosphatase, lambda phosphatase and inhibitors, or inhibitors alone. C, APC association to Cks1-coupled beads changes through the cell cycle. Wild-type (ADR1389) and *CDC28-VF* (ADR1252) were grown overnight at 30°C in YPD to log phase, arrested in G1 with alpha factor (1 μg/ml), and at t = 0 (alpha factor) the cells were released from the G1 arrest. At t = 75, alpha factor (1.5 μg/ml) was added back to the cultures to rearrest the cells in the next G1. A parallel sample was arrested in mitosis with nocodazole (10 μg/ml). Samples were taken at the indicated times and processed for Western blots (top) or for Cks-coupled bead pulldowns (bottom). The arrow indicates that in wild-type cells when Clb2 levels peak (t = 75), Cdc16 and Cdc27 phosphorylation increases. The bracket indicates that in *CDC28-VF* cells when Clb2 levels are peaking, Cdc27 phosphorylation decreases. D, An APC-containing Cdc27-5A does not bind to Cks1-coupled beads. The strains in Fig. 3 B and *CDC28-VF pGAL-MPS1* (KH181) were grown overnight in YEP + 2% raffinose at 23°C to log phase and then transferred to YEP + 2% galactose for 4 h to arrest the cells in mitosis by Mps1 overexpression. Samples were taken and processed for Western blots (cell lysate), immunoprecipitation with anti-cdc26 antibodies, or Cks1-coupled bead pulldowns as described in Materials and Methods. Equal amounts of lysates were used for the Cks1-coupled bead pulldown and the anti-Cdc26 immunoprecipitation, though a longer exposure is shown for the Cks1-coupled bead pulldown. Clb2 is shown as a loading control. E, Mitotic Cdc28 activity is required for APC phosphorylation. Wild-type (ADR376), *CDC28-VF* (ADR509), *cdc28-1N* (ADR483), *cdc28-4* (ADR842), *clb2*Δ (ADR313), and *cks1-38* (ADR1767) were grown overnight in YEP + 2% glucose at 23°C to log phase and arrested in mitosis with nocodazole (10 μg/ml) for 3.5 h. Samples were taken at the indicated times and processed for Western blots. Clb3 is shown as a loading control.

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-

berg and Hershko, 1999). Fig. 4 A shows that the APC from mitotically arrested yeast cells binds to Cks1-coupled beads. Comparing Western blots of the material recovered from wild-type and *CDC28-VF* cells reveals that less APC from *CDC28-VF* cells binds to Cks1-coupled beads. 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 *CDC28-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 *CDC28-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 *CDC28-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 *CDC28-VF* cells. These differences suggest that mitotic phosphorylation by Cdc28 is required for APC binding to Cks1-coupled beads (Sudakin et al., 1997). Although the difference in APC phosphorylation between wild-type and *CDC28-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 (*CDC28-VF*, *cdc28-1N*, *clb2*Δ, and *cks1-38*) are hypersensitive to checkpoint arrest caused by overexpression of Mps1, whereas a mutant that primarily affects G1 activity (*cdc28-4*) 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 *clb2*Δ, *cdc28-1N*, and *cks1-38* all have reduced levels and resemble *CDC28-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. APC 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 (*CDC16-6A*, *CDC23-A*, or *CDC27-5A*). 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

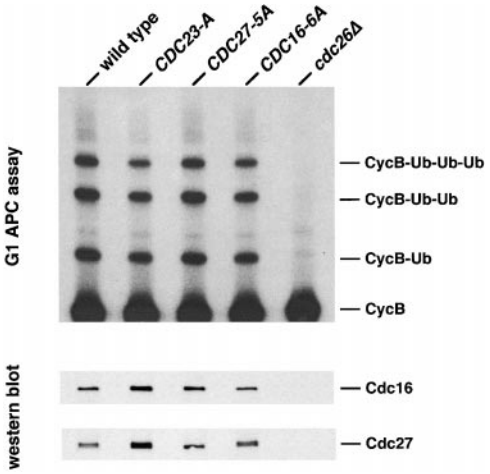


Figure 5. The alanine-substituted APC has normal G1 APC activity. The strains described in Fig. 3 C were grown overnight at 30°C in YEP + 2% glucose to log phase, and arrested in G1 with alpha factor (1 μg/ml) for 3 h. The cells were harvested, lysed, and the APC was immunoprecipitated with anti-Cdc26 antibodies, and the in vitro ubiquitination activity of the immunoprecipitates was measured. The substrate for the in vitro ubiquitination is an iodinated NH_2-terminal fragment of sea urchin Cyclin B (CycB). Western blotting of the immunoprecipitates (bottom) shows that equal amounts of Cdc16 and Cdc27 are present in the APC isolated from each of the strains.

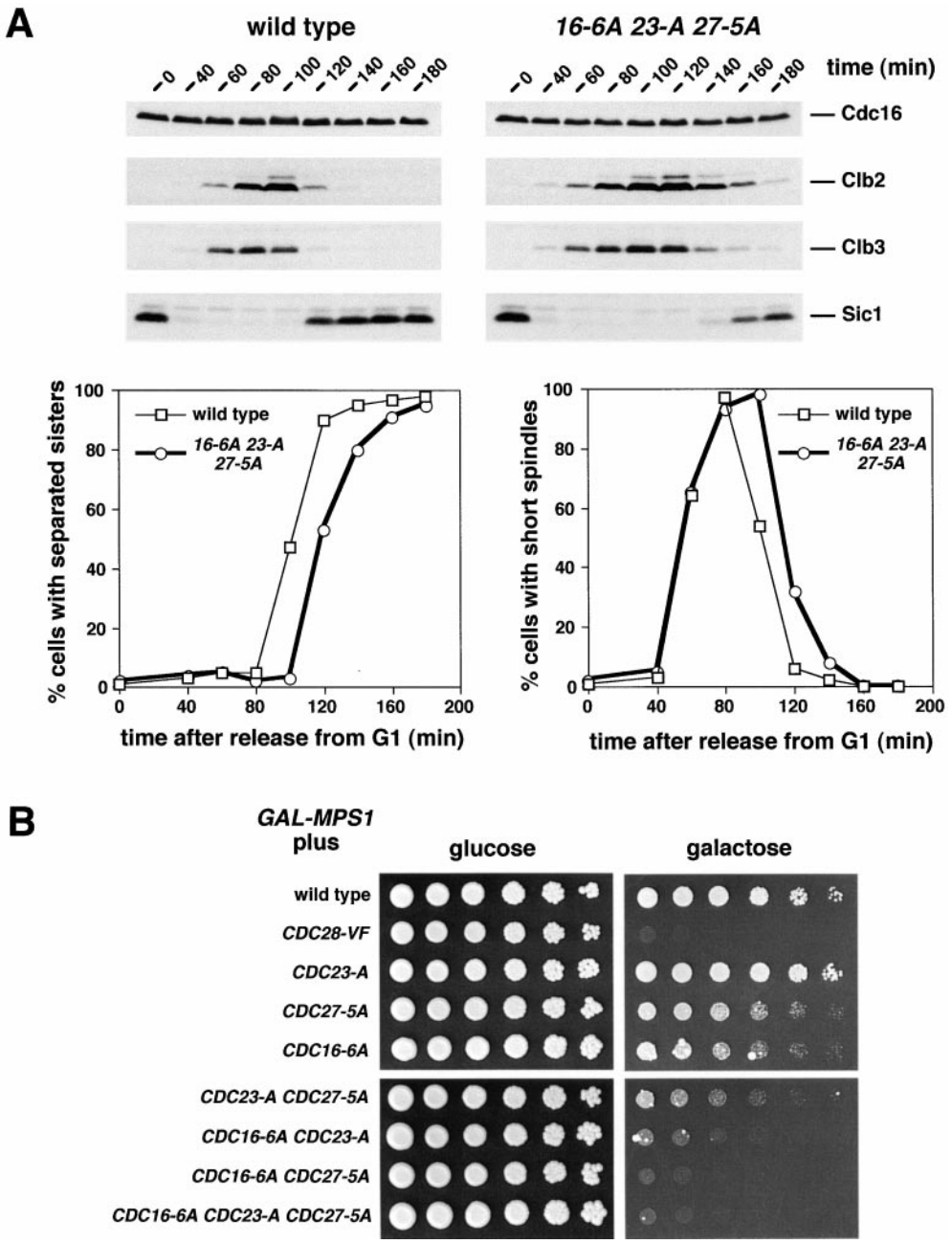


Figure 6. The alanine-substituted APC delays in mitosis and is sensitive to spindle checkpoint-dependent arrest. A, Wild-type (ADR2061) and *CDC16-6A CDC23-A CDC27-5A* (ADR2064) were grown overnight at 23°C in YPD to mid log phase, arrested in G1 with alpha factor (1 μg/ml), and at t = 0 the cells were released from the G1 arrest. At t = 80, alpha factor (1.5 μg/ml) was added back to the cultures to rearrest the cells in the next G1. Top, Samples were taken at the indicated times and processed for Western blots. Bottom left, Sister chromatid separation was scored by counting the number of fluorescent spots (one or two) of green fluorescent protein (GFP)-lacI bound to 256 tandem repeats of *lacO* integrated at the *LEU2* locus. Bottom right, Spindles were visualized by indirect immunofluorescence of formaldehyde fixed cells probed with an antialpha-tubulin antibody. B, All strains contain *pGAL-MPS1*. Wild-type (KH153), *CDC28-VF* (KH181), *CDC23-A* (ADR-1973), *CDC27-5A* (ADR-1974), *CDC16-6A* (ADR-1975), *CDC23-A CDC27-5A* (ADR1976), *CDC16-6A CDC23-A* (ADR1977), *CDC16-6A CDC27-5A* (ADR1978), and *CDC16-6A CDC23-A CDC27-5A* (ADR1979) were grown to saturation for 2 d in YEP + 2% glucose at 30°C, diluted tenfold, and fourfold serial dilutions were prepared in a multiwell dish and spotted onto YEP + 2% glucose (left) or YEP + 2% galactose (right). The plates were incubated at 30°C for 2 d.

APC activity. In addition, cells carrying alanine-substituted APC subunits show no obvious growth defects at any temperature.

We asked if the alanine substitutions in the APC, 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). Clb2 and Clb3 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 APC phosphorylation sites also causes an increased sensitivity to spindle checkpoint arrest caused by Mps1 overexpression (Hardwick et al., 1996; Rudner et al., 2000). Serial dilutions of wild-type, *CDC28-VF*, mutants in single APC 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 mu-

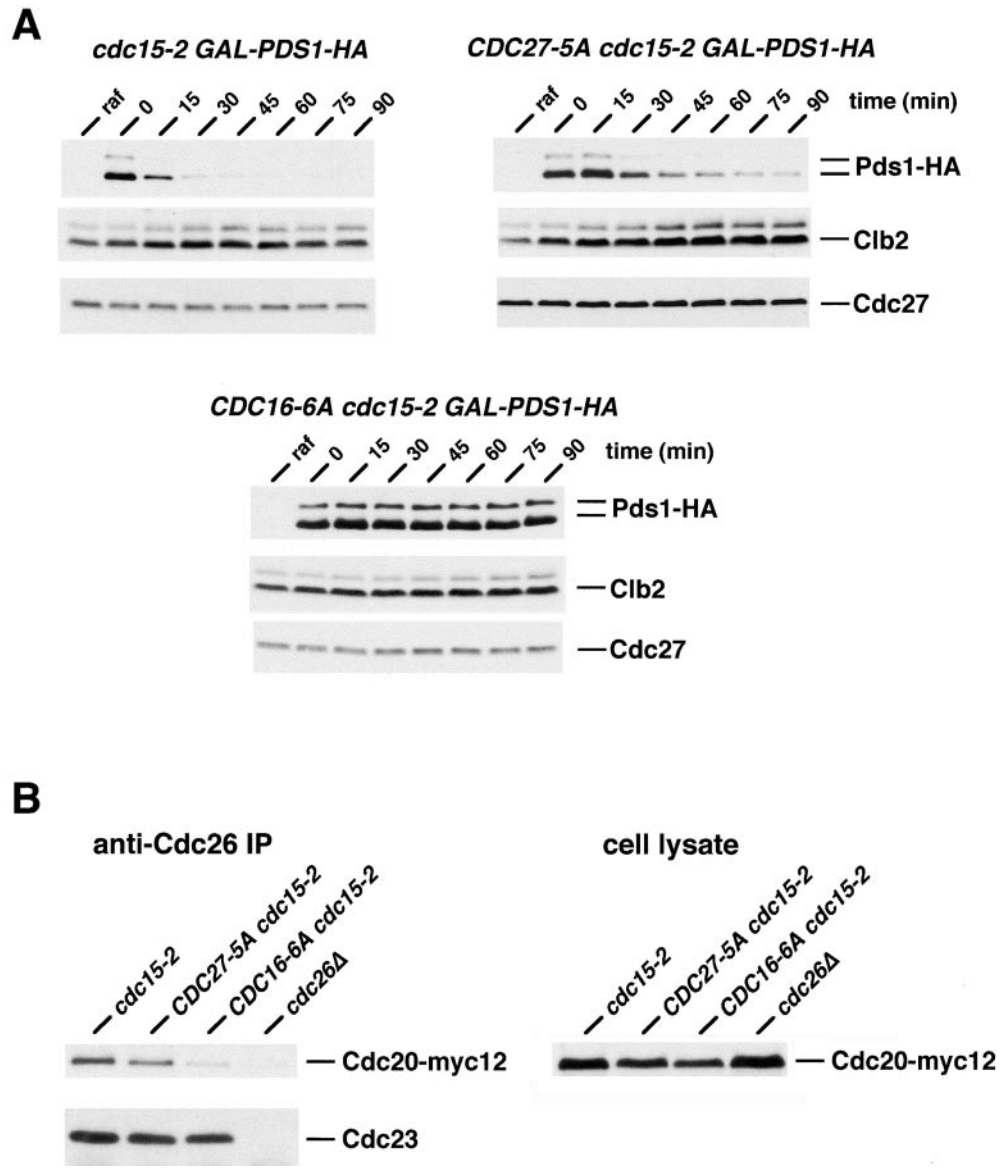
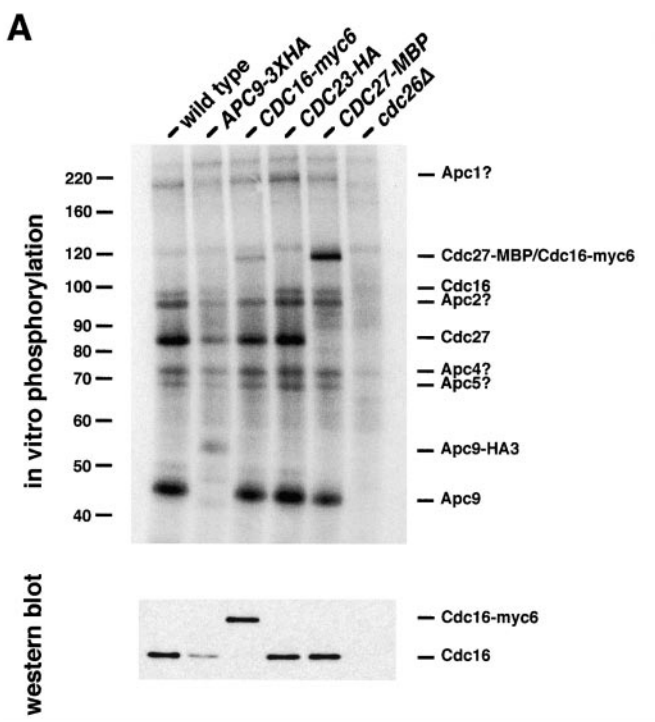


Figure 7. The alanine-substituted APC is defective in Cdc20-dependent APC function. A, Pds1 is stabilized in anaphase in *CDC16-6A* and *CDC27-5A*. *cdc15-2 GAL-PDS1-HA* (ADR1968), *cdc15-2 CDC27-5A GAL-PDS1-HA* (ADR1999), and *cdc15-2 CDC16-6A GAL-PDS1-HA* (ADR2003) were grown overnight at 23°C in YEP + 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* (ADR1790), *cdc15-2 CDC27-5A CDC20-myc12* (ADR1987), *cdc15-2 CDC20-myc12 CDC16-6A* (ADR1990), and *cdc26*Δ CDC20-myc12 (ADR2036) 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 was precipitated with the anti-Cdc26 antibodies (left) and equal amounts of cell lysate were used in the immunoprecipitation (right, cell lysate). *cdc26*Δ, 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).

tants creates a phenotype similar to that of *CDC28-VF*. 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 nonphosphorylatable 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 nonphosphorylatable APC mutants in anaphase (using the *cdc15-2* mutant), induced Pds1 expression from the *GAL1* 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 *clb2*Δ 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. 7 A).

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



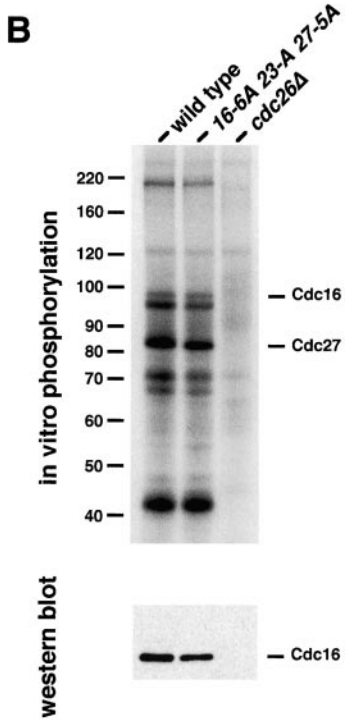


Figure 8. Cdc5 phosphorylates the APC in vitro. A, The APC was isolated from the following strains as described in Fig. 1: Wild-type (ADR376), *APC9-HA3* (ADR2042), *CDC16-myc6* (K6180), *CDC23-HA* (SLJ378), *CDC27-MBP* (ADR1705), and *cdc26*Δ (LH307). The immunoprecipitates were treated with purified His_6-HA-Cdc5 and γ[^{32}P]ATP, washed to remove phosphorylated His_6-HA-Cdc5, and run on a polyacrylamide gel that was subjected to autoradiography (top) or Western blotting (bottom). The Western blot shows that similar amounts of APC were precipitated with the anti-Cdc26 antibody from all strains except *APC9-HA3*, which does not fully complement a *apc9*Δ. B, The APC was isolated from wild-type (ADR376), *CDC16-6A CDC23-A CDC27-5A* (ADR2032), and *cdc26*Δ (LH307), and phosphorylated by purified His_6-HA-Cdc5.

al., 2000). We arrested *cdc15-2*, *cdc15-2 CDC16-6A*, and *cdc15-2 CDC27-5A* cells in anaphase, immunoprecipitated the APC with anti-Cdc26 antibodies, and examined the amount of associated Cdc20. The association of Cdc20 to the APC 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 APC kinase in vivo (Lahav-Baratz et al., 1995; Kotani et al., 1998; Patra and Dunphy, 1998; Shteinberg and Hershko, 1999; Shteinberg et al., 1999). Since we find that APC phosphorylation is reduced in a *cdc5-1* mutant (Fig. 2 C), we tested whether purified recombinant Cdc5 could phosphorylate the APC in vitro. Like Cdc28, Cdc5 phosphorylates Cdc16, Cdc27, and Apc9 (Fig. 8 A), but unlike Cdc28, appears to not phosphorylate Cdc23. Cdc5 also phosphorylates proteins that have the molecular weights of several other APC subunits (Apc1, -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 APC 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 APC. In our hands, however, Cdc5's ability to phosphorylate the APC does not increase when the kinase is pretreated with purified Cdc28/Clb2/Cks1 complexes (data not shown).

We next tested whether purified Cdc5 can phosphorylate the alanine-substituted APC. 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., 1994). In contrast to their effect on phosphorylation by Cdc28, the APC phosphorylation site mutants had no effect on in vitro phosphorylation of the APC by recombinant Cdc5 (Fig. 8 B). This observation makes it likely that the reduced in vivo APC phosphorylation seen in *cdc5-1* cells is an indirect effect of reduced Cdc5 activity, rather than a direct in vivo phosphorylation of these APC subunits by Cdc5.

Discussion

We have shown that the budding yeast APC 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/Clb2/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 APC. We have also shown that Cdc5 affects APC phosphorylation in vivo and can catalyze APC phosphorylation in vitro. Our analysis of APC 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; Yamada et al., 1997; Patra and Dunphy, 1998; Kotani et al., 1999). Although APC phosphorylation has been shown to activate the Cdc20-dependent APC in mammalian tissue culture and clam egg extracts (Kotani 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 APC phosphorylation. Although 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-Baratz et al., 1995; Vorlaufer and Peters, 1998).

In one report, Plk has been identified as the major kinase of the mammalian APC (Kotani et al., 1998), although others have argued that this role is played by Cdc2 (Lahav-Baratz 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/Clb2/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/Clb/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 *CDC27-5A* and *CDC16-6A* 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 *CDC16-6A CDC23-A CDC27-5A* 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 the 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/Clb2/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 *CDC16-6A CDC23-A CDC27-5A* 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 *CDC27-5A* mutant. Despite this in vivo finding, we do see reduced in

vitro phosphorylation of Cdc16 and Cdc23 in an APC containing Cdc27-5A (Fig. 3 C).

Mutants that affect the mitotic form of Cdc28 have reduced levels of phosphorylation of the APC, 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 APC 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 APC phosphorylation because one Cdc28/Clb2/Cks1 complex may bind persistently to the APC. Once bound to the APC, this single complex might be responsible for multiple phosphorylations. If the steady state phosphorylation of the APC is determined by the balance between phosphorylation by Cdc28 and dephosphorylation by protein phosphatases, and Cdc28 remains bound to the APC, a drop in specific activity of Cdc28 would reduce the phosphorylation and activity of the APC.

How do cells escape from mitosis? If activating Cdc28/Clb/Cks1 complexes activates the Cdc20-dependent APC, which in turn triggers chromosome segregation, how do cells ensure that the lag between activating Cdc28 and activating the Cdc20-dependent APC is long enough to assemble a spindle and align chromosomes on it? Although 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 APC. Another possible mechanism is regulating the abundance of Cdc20. High levels of *CDC20* transcripts are restricted to mitotic cells and APC-dependent proteolysis restricts the abundance of Cdc20 (Weinstein, 1997; Kramer et al., 1998; Prinz et al., 1998; Shirayama et al., 1998). None of these forms of regulation exist in early frog embryos, where Cdc20 (Fizzy) levels are constant through the cell cycle and spindle depolymerization does not induce mitotic arrest (Minshull et al., 1994; Lorca et al., 1998). In addition, overexpressing Cdc20 in budding yeast raises the level of Cdc20 mRNA and protein, but does not advance the exit from mitosis, suggesting that other mechanisms must exist to regulate Cdc20-dependent APC activity (Prinz et al., 1998). Regulating the rate of Cdc28-catalyzed APC phosphorylation provides an additional mechanism. If this phosphorylation were slow relative to spindle assembly, most cells would manage to align their chromosomes on the spindle before activating the Cdc20-dependent APC, which in turn induces Pds1 destruction and anaphase.

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

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