Abstract. The activity of the cyclin-dependent kinase 1 (Cdk1), Cdc28, inhibits the transition from anaphase to G1 in budding yeast. CD C28-T 18V, Y 19F (CDC28-VF), a mutant that lacks inhibitory phosphorylation sites, delays the exit from mitosis and is hypersensitive to perturbations that arrest cells in mitosis. Surprisingly, this behavior is not due to a lack of inhibitory phosphorylation or increased kinase activity, but reflects reduced activity of the anaphase-promoting complex (APC), a defect shared with other mutants that lower Cdc28/Clb activity in mitosis. CDC28-VF has reduced Cdc20-dependent A PC activity in mitosis, but normal H ct1-dependent A PC activity in the G1 phase of the cell cycle. The defect in Cdc20-dependent A PC activity in CDC28-VF correlates with reduced association of Cdc20 with the A PC. The defects of CDC28-VF suggest that Cdc28 activity is required to initiate the metaphase to anaphase transition and initiate the transition from anaphase to G1 in budding yeast.

Key words: anaphase-promoting complex • H ct1 • Cdc20 • Pds1 • sister chromatid separation

Introduction

To exit mitosis, cells must accomplish two tasks: chromosome segregation and inactivation of complexes between mitotic cyclins and cyclin-dependent kinase 1 (Cdk1)3, known as Cdc28 in budding yeast and Cdc2 in other eu karyotes, which allows the cell cycle to progress into G1. Both steps require the activity of the anaphase-promoting complex (A PC) or cyclosome, a multiprotein complex that is required for the ubiquitination of cyclin and other unstable substrates (K ing et al., 1995; Sudakin et al., 1995; Zachariae and Nasmyth, 1996, 1999; Y anagida et al., 1999). The activity of the A PC depends on its interaction with two WD-40 proteins, Cdc20 (Sethi et al., 1991; Sigrist et al., 1995; Visintin et al., 1997; Lorca et al., 1998) and H ct1 (Schwab et al., 1997; Sigrist and Lehner, 1997; Visintin et al., 1997; Y amaguchi et al., 1997; K itamura et al., 1998). Cdc20 initiates the metaphase to anaphase transition by inducing ubiquitination of the anaphase inhibitor, Pds1 (Cohen-Fix et al., 1996; Funabiki et al., 1997). This reaction causes Pds1 degradation and sister chromatid separation (Funabiki et al., 1996; Ciosk et al., 1998). CDC28-VF is a mutant that lacks inhibitory phosphorylation sites, reducing the kinase activity of Cdc28 and further increasing the activity of the A PC (Zachariae et al., 1998; Jaspersen et al., 1999). This inhibition is opposed by the phosphatase Cdc14, which de-phosphorylates H ct1, initiating a positive feedback loop that drives the cell into G1 (Visintin et al., 1998; Jaspersen et al., 1999). A s H ct1 activity rises, the rate of Clb destruction increases, reducing the kinase activity of Cdc28 and further activating H ct1. The CDK inhibitor, Sic1 (M endenhall, 1993; Donovan et al., 1994), which inhibits Cdc28/Clb activity in mitosis, is required for the ubiquitination of cyclin, but is not required in the G1 phase of the cell cycle. The defect in Cdc20-dependent A PC activity in mitosis, but normal H ct1-dependent A PC activity in the G1 phase of the cell cycle. The defect in Cdc20-dependent A PC activity in CDC28-VF correlates with reduced association of Cdc20 with the A PC. The defects of CDC28-VF suggest that Cdc28 activity is required to initiate the metaphase to anaphase transition and initiate the transition from anaphase to G1 in budding yeast.

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complexes, also participates in this feedback loop, since both its transcription and stability are inhibited by Cdc28/Cib activity (Amon et al., 1997; Toyn et al., 1997; Vissintin et al., 1998).

Does Cdc28 also play a role in inducing anaphase? In frog and clam egg extracts, activation of the A PC depends on active Cdc2/Cyclin B complexes (Felix et al., 1990; Hersko et al., 1994; M inshull et al., 1994; Shieh and Hershko, 1999). This activating role might be explained by the finding that Cdc2/Cyclin B can phosphorylate subunits of the A PC (Patra and D unphy, 1998; K otani et al., 1999), and this phosphorylation is correlated with activating the A PC and binding of Fizzy, the Cdc20 homologue in frogs (Peters et al., 1996; Fang et al., 1998b; K otani et al., 1998; Shieh and Hershko, 1999). In embryonic cell cycles, no Cdc1 homologue is present (Sigrist and Lehner, 1997; L orca et al., 1998), suggesting that Cdc20-dependent A PC activity targets both Pds1 homologues and mitotic cyclins for destruction. In budding yeast, the inability of Cdc20 to catalyze the complete destruction of mitotic cyclins means that Cdc1 or Sic1 is required for cells to exit from mitosis (Schwab et al., 1997; Vissintin et al., 1997). Since both Cdc1 and Sic1 are inhibited by Cdc28, it has been difficult to ask whether a previous step in the exit from mitosis requires Cdc28.

Cdc28/Cib activity is also regulated by inhibitory phosphorylation on residues tyrosine 19, and possibly threonine 219 (Amon et al., 1992; Sorger and Murray, 1992). Inhibitory phosphorylation on residues tyrosine 19, and possibly threonine 219, of Cdc28, may be induced by the checkpoints that detect unreplicated or damaged DNA (Gould and Nurse, 1989; Enoch and Hershko, 1999). This activating role might be explained by the finding that Cdc2/Cyclin B can phosphorylate subunits of the A PC (Patra and Dunphy, 1998; K otani et al., 1999), and this phosphorylation is correlated with activating the A PC and binding of Fizzy, the Cdc20 homologue in frogs (Peters et al., 1996; Fang et al., 1998; K otani et al., 1998; Shieh and Hershko, 1999). In embryonic cell cycles, no Cdc1 homologue is present (Sigrist and Lehner, 1997; L orca et al., 1998), suggesting that Cdc20-dependent A PC activity targets both Pds1 homologues and mitotic cyclins for destruction. In budding yeast, the inability of Cdc20 to catalyze the complete destruction of mitotic cyclins means that Cdc1 or Sic1 is required for cells to exit from mitosis (Schwab et al., 1997; Vissintin et al., 1997). Since both Cdc1 and Sic1 are inhibited by Cdc28, it has been difficult to ask whether a previous step in the exit from mitosis requires Cdc28.

Cdc28/Cib activity is also regulated by inhibitory phosphorylation on residues tyrosine 19, and possibly threonine 219 (Amon et al., 1992; Sorger and Murray, 1992; B oother et al., 1993). Tyrosine 19 is phosphorylated by Swe1 (the homologue of Wee1 in fission yeast) and dephosphorylated by Mih1 (the homologue of Cdc25 in fission yeast; Amon et al., 1992; Sorger and Murray, 1992). Inhibitory phosphorylation on residues tyrosine 19, and possibly threonine 219, of Cdc28, may be induced by the checkpoints that detect unreplicated or damaged DNA (Gould and Nurse, 1989; Enoch and Hershko, 1999). This activating role might be explained by the finding that Cdc2/Cyclin B can phosphorylate subunits of the A PC (Patra and Dunphy, 1998; K otani et al., 1999), and this phosphorylation is correlated with activating the A PC and binding of Fizzy, the Cdc20 homologue in frogs (Peters et al., 1996; Fang et al., 1998; K otani et al., 1998; Shieh and Hershko, 1999). In embryonic cell cycles, no Cdc1 homologue is present (Sigrist and Lehner, 1997; L orca et al., 1998), suggesting that Cdc20-dependent A PC activity targets both Pds1 homologues and mitotic cyclins for destruction. In budding yeast, the inability of Cdc20 to catalyze the complete destruction of mitotic cyclins means that Cdc1 or Sic1 is required for cells to exit from mitosis (Schwab et al., 1997; Vissintin et al., 1997). Since both Cdc1 and Sic1 are inhibited by Cdc28, it has been difficult to ask whether a previous step in the exit from mitosis requires Cdc28.

Our previous work suggested that inhibitory phosphorylation of Cdc28 might aid in mitotic exit. Cdc55Δ, cells, which lack a B subunit of protein phosphatase 2A (PP2A; H ealy et al., 1991), are spindle checkpoint defective and have increased phosphorylation on tyrosine 19 of Cdc28 (Healy et al., 1991), are spindle checkpoint defective and have increased phosphorylation on tyrosine 19 of Cdc28 (Healy et al., 1991). Inhibitory phosphorylation of Cdc28 is required for the bud emergence or morphogenesis checkpoint, which delays mitosis in cells that have not budded (L ew and R eed, 1993).

Table I lists the strains used in this work. All strains are derivatives of the W303 strain background (W303-1a; R odney R othstein, Columbia U niversity, 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 were confirmed by PCR or by mutant phenotype. The sequences of all oligonucleotide primers used in this study are available upon request. The strains T G1 and D H5 were used for all bacterial manipulations.

The strains used for the crosses in Table II were: JM 434 (CDC28-VF), JM 469 (CDC28-V), JM 467 (CDC28-F), A D R 1541 (CDC28-AF), A D R 2035 (CDC28-F8RG), K H 208 (swelα), A D R 484 (cdc28-1N), A D R 840 (cdc28-4), A D R 314 (cib2Δ), A D R 719 (cib2Δ3), A D R 1147 (cdc61-1), L H 226 (doc1-1), L H 125 (cdc28-1), A D R 1435 (hct1Δ), J C 126 (cdc5-1), A D R 1298 (cdc13-1), and K 193 (cdc15-2). For all crosses, at least 22 tetrads were analyzed.

**Materials and Methods**

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cloned into pA R125 cut with SpeI/XmnI, to create pA R127, which replaces the entire HCT1 open reading frame with H135. The EcoRI/NheI fragment of pA R127 was used to transform yeast.

CD 20 was tagged at the NH2 terminus. A cassette containing 12 myc tags (pL H71) was inserted into pCM4 (Hwang et al., 1998) cut with EcoRI.
Table II. Genetic Interactions of CDC28-VF

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sl, Synthetic lethal; ND, not determined; +, viable.

cus, creating a duplication of CDC20 and CDC20-12myc marked with the URA3 gene. This strain was grown on 5-fluoroorotic acid to select against the URA3 gene, and the resulting colonies were screened by Western blot for the presence of CDC20-12myc to create L.H.H.371. This strain was crossed to the appropriate strains to create those used in this study.

**Physiology**

Stock solutions of inhibitors were: 10 mg/ml alpha factor (Biosynthesis); 30 mg/ml nocodazole (Sigma-Aldrich). All stocks were stored at -20°C in DMSO.

For microcolony assays, cells were grown to mid-log phase in yeast extract and peptone (YPEP) + 2% raffinose, spotted onto a YEP + 2% galactose plate, and unbudded cells were picked out into a grid with a dissecting needle. The number of cells in each microcolony was counted at different times after incubation at 30°C. Each bud is counted as a cell, and the original cells that did not bud are not included in the analysis.

For serial dilution and spotting, cells were prepared in a multiwell dish or a microtiter plate, and using a multiprong applicator, ~10 μl of each strain and its dilutions were spotted onto various plates and incubated at either 23 or 30°C.

Sister chromatid separation was visualized using a fusion of green fluorescent protein (GFP) and lacI, creating a duplication of pRS426, which had been integrated at specific locations on yeast chromosomes (Straight et al., 1996). Small samples of cells were harvested at the indicated times and either fixed in 4% paraformaldehyde at 4°C, and then washed twice in 0.1 M KPO4, pH 7.4. The cells were then mounted on slides and viewed by fluorescence microscopy (Nikon). In all experiments, a minimum of 200 cells were counted per time point.

The following antibodies were used in Western blots: 9E10 ascites (BabCO) was used at a dilution of 1:1,000 in PBST + 0.2% NaN3 after blocking in 4% nonfat dried milk in PBST. Afinity-purified rabbit polyclonal anti-Cib2 and anti-Cib3 antibodies (Kellok and Murnay, 1995) were used at a dilution of 1:1,200 in blocking buffer (2% BSA, PBST, 0.5M NaCl, 0.02% NaN3). Rabbit polyclonal anti-Sic1 serum (a gift of Mike Mendenhall, University of Kentucky, Lexington, KY) was used at a dilution of 1:1,000 in blocking buffer (4% nonfat dried milk, 2% BSA, PBST, 0.02% NaN3 + 10 μg/ml cell lysate made from sicΔ1 cells (J M 408, made with MDM p203; a gift of M ike M endenhall). 12CA5 ascites (BabCO) was used at a dilution of 1:1,000, rabbit polyclonal anti-Cdc16 and anti-Cdc23 (Lamb et al., 1994) were used at 1:2,000, and anti-Cdc27 (Lamb et al., 1994) was used at 1:2,500. These four antibodies were all diluted in blocking buffer (4% nonfat dried milk, PBST, 0.02% NaN3).

The remaining lysate was used for immunoprecipitation. 2–20 mg of lysate per sample was used depending on the experiment. 0.33–3 μg of antibody was added to the lysate and incubated on ice for 20 min. Samples were then centrifuged in a microfuge at 14,000 rpm for 5 min at 4°C and transferred to 10–15 μl of 2X SDS sample buffer after 5 min at 4°C. Western blots were probed with rabbit polyclonal anti-A Clb4-YP (Schleifer and Schuel; Minshull et al., 1996). Blots were stained with Ponceau S to confirm transfer and equal loading of samples, and then blocked for 30 min in antibody-specific blocking buffer (see below). All antibodies were incubated overnight at 4°C or 2 h at 25°C. A ferter washing in PBST (PBS + 0.1% Tween-20, M anilat is et al., 1982), the blots were then incubated in HRP-conjugated anti-rabbit or anti-mouse antibodies (A mersham Pharmacia Biotech) at a 1:5,000 dilution in PBST for 30 min at 25°C, washed again, incubated in ECL detection reagents (A mersham Pharmacia Biotech) or Renaissance reagents (NE N Life Science Products), using the manufacturer’s instructions, and then exposed to X-O M A T film (K odak).

For histone H1 kinase assays, 0.33 μg of anti-Cib2 or anti-Cib3 antibody was used for immunoprecipitation in 1–5 mg of cell lysate. A fter immunoprecipitation, the beads were washed three times in kinase 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 twice in kinase buffer (80 mM Na-β-glycerophosphate, pH 7.4, 15 mM MgCl2, 20 mM EGTA). A ll washes were performed on ice. Kinase reactions were performed in 15 μl of kinase buffer containing 1 mM DTT, 25 μM ATP, 2.5 μM histone H1 (U pstate Biotechnology) and 1 μC of γ-32P[ATP (A mersham Pharmacia Biotech) and were incubated for 15 min at 25°C. Reactions were stopped by adding 15 μl of 2 X SDS sample buffer and heating samples to 99°C for 5 min. Samples were run on a 15% polyacrylamide gel, stained, and dried. Kinase gels were quantified using a Molecular Dynamics PhosphoImager and ImageQuant software.

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**PhosphoTyrosine Detection**

For antiphosphotyrosine detection, 2 μg of 12CA5 antibody was used to immunoprecipitate Cdc28-HA or Cdc28-VF-HA from 15–20 mg of cell ly-
tates were then processed for Western blots as described above.

For APC assay, 1 µg of rabbit polyclonal anti-Cdc26 antibody (Hwang and Murray, 1997) was used to immunoprecipitate the APC from 5 mg of cell lysate. The APC assay was conducted as previously described (Charles et al., 1998), except that cells were lysed in the lysis buffer described above, the beads were washed in APC bead buffer (250 mM NaCl, 50 mM Tris-Cl, pH 7.4, 50 mM NaF, 5 mM EGTA, 5 mM EDTA, 0.1% NP-40, 1 mM DTT) three times, and then twice in QA + NaCl buffer (20 mM Tris-Cl, pH 7.6, 100 mM NaCl, 1 mM MgCl2). 25 mM of purified Hcl1 (Jasperse et al., 1999) was added to some samples during the ubiquitination reaction.

**Results**

**CDC28-VF Impairs Mitotic Exit**

Since CDC28-VF suppresses the checkpoint defect of cdc55A (Minshull et al., 1996), we wondered if the CDC28-VF mutant alone might have difficulty leaving mitosis. Fig. 1 A shows that progression through mitosis is delayed in CDC28-VF. Wild-type and CDC28-VF cells were arrested in G1 by the mating pheromone alpha factor and released into the cell cycle. Once cells had budded, alpha factor was readded to arrest cells that had completed the cycle. This regimen allows us to look clearly at one synchronous cell cycle. CDC28-VF cells show a 30-min delay in the degradation of the anaphase inhibitor Pds1 and sister chromatid separation. Cib2 proteolysis and the fall in Cib2-associated kinase activity are delayed by >30 min. These delays can be partially attributed to a 15-min delay in Cib2 accumulation, but the persistence of peak levels of Cib2 for at least 60 min clearly reflects an additional defect in CDC28-VF. In addition, mitotic entry is not delayed because short spindles, a marker for mitotic entry, appear at the same time in CDC28-VF and wild-type cells (data not shown). CDC28-VF does not delay exit from G1, since the CKI inhibitor, Sic1 (Schwob et al., 1994), disappears at the same time (30 min) in wild-type and CDC28-VF.

Although CDC28-VF delays passage through mitosis, the doubling time of CDC28-VF cells is nearly identical to that of wild-type cells (data not shown). This apparent paradox can be explained by the fact that CDC28-VF cells exit mitosis at a larger cell size than wild-type cells, and therefore have to grow less in G1 to reach the critical cell size needed to pass Start, the cell cycle transition that commits them to replicating their DNA (Johnston et al., 1977). Thus, the increase in time spent in mitosis is made up by a decrease in time spent in G1.

To see if the mitotic delay in CDC28-VF is due to difficulty exiting mitosis, we examined cells that were recovering from activation of the spindle checkpoint. Wild-type and CDC28-VF cells were arrested in mitosis by treating them with nocodazole (an inhibitor of microtubule polymerization) for three hours and then released from this arrest into fresh medium containing alpha factor to arrest them in G1 as they left mitosis. Wild-type cells degrade Clb2, inactivate Clb2-associated kinase, and separate their sisters within 90 min of removing nocodazole (Fig. 1 B). CDC28-VF cells, however, take 150-180 min to fully escape from the nocodazole arrest.

Since CDC28-VF cells are delayed in exiting mitosis, we investigated how they responded to a prolonged mitotic arrest caused by the spindle checkpoint. We examined the response to overexpressing the protein kinase Mps1, which arrests cells in mitosis by activating the checkpoint, but does not damage the spindle (Hardwick et al., 1996). A 24-hr over expression of Mps1 delays mitosis, divide, and resume proliferating. In contrast, CDC28-VF cells overexpressing Mps1 cannot proliferate (Fig. 2 A); when individual cells are followed microscopically, many never divide, and the remainder go through only one or two divisions (Fig. 2 B and data not shown). The cell cycle arrest and eventual lethality are completely suppressed by the mad3Δ mutation, which inactivates the spindle checkpoint (Li and Murray, 1991; Hardwick et al., 2000). mad3Δ and mad3Δ CDC28-VF cells divide as if there were no activation of the checkpoint. These results show that CDC28-VF cells, unlike wild-type, cannot escape from mitosis in the presence of constant stimulation of the spindle checkpoint. CDC28-VF cells are also sensitive to other perturbations that activate the spindle checkpoint, including the presence of short linear chromosomes (Wells and Murray, 1996) and mutations that damage the spindle (Hardwick et al., 1999). Like Mps1 overexpression in CDC28-VF, these treatments are lethal and cause long delays in mitosis (data not shown).

**The Mitotic Defect of CDC28-VF Is Not Caused by a Lack of Inhibitory Phosphorylation**

We initially observed that CDC28-VF and cdc55A have opposite effects on the exit from mitosis, consistent with the idea that inhibitory phosphorylation of Cdc28 aids exit from mitosis. More careful examination reveals that the mitotic exit defect in CDC28-VF is not due to effects on Cdc28 phosphorylation. Fig. 3 A shows serial dilutions of a panel of mutants, spotted onto galactose-containing plates, which induce Mps1 overexpression, or glucose-containing plates, which do not. swelΔ cells lack the tyrosine kinase that phosphorylates Cdc28 (Booher et al., 1993). Like CDC28-VF cells, they have no phosphotyrosine present on Cdc28 (Fig. 3 B), but unlike CDC28-VF or CDC28-F, they are no more sensitive to Mps1 overexpression than wild-type cells. Since another kinase might phosphorylate the threonine 18 in swelΔ cells, we investigated the behavior...
of swe1Δ CDC28-V cells, which should lack all inhibitory phosphorylation. swe1Δ CDC28-V resemble CDC28-V cells, both being only slightly more sensitive to Mps1 overexpression than wild-type cells. mih1Δ cells, which like cdc55Δ cells have increased inhibitory phosphorylation on Cdc28 (Fig. 3 B), do not have a spindle checkpoint defect as judged by their sensitivity to Mps1 overexpression or to microtubule depolymerizing agents (Fig. 3 A and data not shown).

In principle, the relative insensitivity of swe1Δ to Mps1 overexpression could be explained by the existence of other kinases that phosphorylate tyrosine 19 of Cdc28. We do not believe such a kinase exists. We have never detected phosphotyrosine on Cdc28 in swe1Δ or swe1Δ mih1Δ cells (Fig. 3 B and data not shown), and mih1Δ cells show a 15-min delay in entering mitosis that is completely suppressed by deleting SWE1, suggesting that Swe1 is the only kinase responsible for inhibiting Cdc28 (data not shown). In addition, if such a kinase existed, we would expect CDC28-AF (Amon et al., 1992; Sorger and Murray, 1992), which substitutes alanine at position 18 of Cdc28 rather than valine, to behavior identically to CDC28-V. However, CDC28-AF...
behaves like CDC28-F, both of which are less sensitive to Mps1 overexpression than CDC28-VF (Fig. 3 A).

We favor the idea that the T18V, Y19F substitution causes a phosphorylation-independent defect in Cdc28. Based on the crystal structure of human Cdk2, tyrosine 19 of Cdc28 is adjacent to the gamma phosphate of bound ATP (De Bondt et al., 1993). Thus, CDC28-VF, and to a lesser extent the CDC28-F and CDC28-AF mutations, might affect ATP binding, substrate binding, catalytic activity, or substrate specificity of Cdc28.

**Mitotic Cdc28 Kinase Activity Is Required for Proper Response to the Spindle Checkpoint**

Because the CDC28-VF defect is not due to a lack of inhibitory phosphorylation, we asked if other mutations that affect mitotic Cdc28 activity might share phenotypes with CDC28-VF. cdc28-1N, clb2Δ, and cks1-38 (a mutant in Cks1, a Cdc28-binding protein required for passage through Start and mitosis) are all more sensitive to Mps1 overexpression than wild-type (Fig. 4 A; Piggott et al., 1982; Adwiger et al., 1989; Surana et al., 1991; Tang and Reed, 1993). This phenotype is not seen in cdc28-4, a temperature-sensitive allele of Cdc28 that prevents passage through Start and is primarily defective in the G1 function of Cdc28 (Reed, 1980).

Does a reduction in mitotic Cdc28 activity cause the CDC28-VF phenotype? We have observed that both Clb2- and Clb3-associated kinase activity and total Cdc28-associated kinase activity of CDC28-VF cells is lower than wild-type (Fig. 4 B and data not shown). This is seen both in synchronously cycling cells (Fig. 1, Clb2-associated kinase activity) and in cells arrested by the spindle checkpoint (Fig. 4 B). We estimate the specific activity of a Cdc28-VF/Clb complex is roughly half that of a wild-type Cdc28/Clb complex. Because the difference between wild-type and CDC28-VF is small, it is difficult to know if it is biologically significant. However, an independent substitution in the ATP binding site of Cdc28, CDC28-F88G, has a reduced specific activity in vitro (Bishop et al., 2000) and is as sensitive to overexpression of Mps1 as CDC28-VF (Fig. 4 C).

The mitotic defect of CDC28-VF is semidominant. The heterozygote CDC28/CDC28-VF has an intermediate sensitivity to Mps1 overexpression (data not shown) and overexpression of CDC28-VF in otherwise wild-type cells creates cells that are fully sensitive to overexpressed Mps1 (data not shown and Fig. 5). These results suggest that CDC28-VF is a dominant negative mutant, which competes with the wild-type kinase for substrates, mitotic cyclins, and Cks1. In support of this idea, multiple copies of the CDC28 gene on a 2μ plasmid suppress the lethality of overexpressing Mps1 in a CDC28-VF strain (Fig. 4 D). The semidominant phenotype of CDC28-VF does not reflect haploinsufficiency of Cdc28, since CDC28/cdc28Δ diploids, which contain half as much Cdc28 as CDC28/CDCl28 diploids, do not have a phenotype like CDC28-VF (data not shown).

**CDC28-VF Is Defective in Activating the APC**

Because CDC28-VF impairs the exit from mitosis, crippling other pathways involved in this process might kill CDC28-VF cells. Our inability to make double mutants between CDC28-VF and mutants in the APC supports this idea (Table II). CDC28-VF is synthetically lethal in combi-
The genetic interactions between CDC28-VF and the A PC and its regulators are a mixed blessing. Although they suggest that Cdc28 may help activate the A PC, they prevent us from examining the phenotype of a CDC28-VF APC- double mutant, since it is impossible to create such a mutant. We overcame this difficulty by exploiting the fact that overexpressing Cdc28-VF creates cells that behave phenotypically like CDC28-VF. This overexpression in cdc23-1 cells at the permissive temperature of 23°C is toxic and no cells survive even a brief 1-h pulse of CDC28-VF expression driven by the GAL1 promoter (data not shown).

We overexpressed CDC28 and CDC28-VF in cdc23-1 cells that had been arrested by alpha factor and then released them from the arrest into fresh medium at 23°C, adding alpha factor after budding so that cells will re-arrest when they reach the next G1. Overexpression of CDC28-VF causes a permanent large-budded arrest with high levels of Clb2 and Clb3 (Fig. 5 A). Sic1 levels never rise and Clb2-associated kinase activity never falls. Sister separation and spindle elongation are delayed by 2 h in cells expressing CDC28-VF (Fig. 5 B and data not shown). These observations show that the induction of anaphase is delayed and the transition from anaphase to G1 is completely blocked in cdc23-1 CDC28-VF cells. These phenotypes are not due to activating the spindle checkpoint because neither mad2Δ nor madΔ bub2Δ bypass the arrest (data not shown; Hoyt et al., 1991; Li and Murray, 1991; A lexandru et al., 1999). Therefore, we conclude that CDC28-VF is defective in activating both the CDC20-dependent APC, which triggers anaphase, and the Hct1-dependent APC, which completes cyclin proteolysis.
Figure 4. Mutants with defects in mitotic Cdc28 activity resemble CDC28-VF. A, Defects in mitotic Cdc28 activity are sensitive to spindle checkpoint-dependent arrest. All strains contain pGAL-MPS1. Wild-type (K H153), CDC28-VF (KH181), cdc28-1N (ADR1899), cdc28-4 (ADR1901), clb2Δ (ADR-1606), and cks1-38 (ADR-1903) were grown to saturation for 2 d in YEP + 2% glucose at 23°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 23°C for 2.5 d. B, The specific activity of Cdc28-VF is lower than Cdc28. 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. The cells were then harvested, lysed, and Clb2/Cdc28 and Clb3/Cdc28 complexes were immunoprecipitated, and their histone H1 kinase activity was measured. The Western blot (bottom) shows that equal amounts of Cdc28 are precipitated in the two strains, although the kinase activity (top) of Cdc28-VF is reduced relative to wild-type. The activity of wild-type Cdc28 is reported as 100% for both the anti-Clb2 and anti-Clb3 immunoprecipitates. C, CDC28-F88G behaves like CDC28-VF. All strains contain pGAL-MPS1. Wild-type (K H153), CDC28-VF (KH181), and CDC28-F88G (ADR2034) 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 were spotted onto CSM-trp + 2% glucose (left) or CSM-trp + 2% galactose (right). The plates were incubated at 30°C for 2 d. D, 2μ-CDC28 suppresses CDC28-VF. All strains contain pGAL-MPS1. Wild-type (K H153) or CDC28-VF (K H181) containing either 2μ-CD C28 or an empty 2μ vector were grown to saturation for 2 d in CSM-trp + 2% glucose at 30°C, diluted fivefold, and fourfold serial dilutions were prepared in a multiwell dish and were spotted onto CSM-trp + 2% glucose (left) or CSM-trp + 2% galactose (right). The plates were incubated at 30°C for 2 d.
CDC28-VF Has Normal G1 Hct1-dependent APC Activity

The failure to induce mitotic cyclin proteolysis in cdc23-1 mutants overexpressing Cdc28-VF could reflect the requirement for Cdc20 activity to activate the Hct1-dependent APC (Visintin et al., 1998; Shirayama et al., 1999; Yeong et al., 2000), or it could reflect a Cdc20-independent defect in the activity of Hct1. To distinguish between these possibilities, we asked if CDC28-VF has normal APC activity in G1, a time when all APC activity is Hct1-dependent and Cdc20 is absent (Charles et al., 1998; Prinz et al., 1998; Shirayama et al., 1998; and see below). We immunoprecipitated the APC from alpha factor-arrested cells with antibodies raised against Cdc26, a nonessential component of the APC (Hwang and Murray, 1997), and measured its ability to ubiquitinate an iodinated fragment of sea urchin Cyclin B in a reconstituted ubiquitination assay (Charles et al., 1998). We detected no differences in APC activity in wild-type and CDC28-VF, and the activity of immunoprecipitates from both wild-type and CDC28-VF could be increased by adding recombinant Hct1 (Fig. 6; Jaspersen et al., 1999). In addition, we have shown that Hct1-dependent proteolysis of Clb2 and Pds1 in G1 is normal in CDC28-VF (data not shown and Fig. 7 C). These experiments are consistent with the idea that the CDC28-VF mutant has no direct effect on the activity of the Hct1-dependent APC.

Cdc20 Activates the Cdc20-dependent APC

Cdc20 is required for sister chromatid separation (Shirayama et al., 1998). CDC28-VF shows delays in sister separation (Figs. 1 and 5), suggesting that Cdc20-dependent APC activity is defective in these cells. We have approached this issue in more detail by examining the half life of Pds1, a substrate of the Cdc20-dependent APC (Visintin et al., 1997; Shirayama et al., 1999; Tinker-Kulberg and Morgan, 1999) during anaphase, a time when the Cdc20-dependent APC is thought to be active (Jaspersen et al., 1998). First, we needed to confirm that degradation of Pds1 in anaphase is due to Cdc20 and not Hct1. We arrested wild-type, cdc20-3, and hct1Δ strains in anaphase by over-expressing a nondegradable Clb2 (pGAL-CLB2-Δ176; Surana et al., 1993). These strains also contained an epitope-tagged form of Pds1 replacing the endogenous gene. When all cells had reached anaphase (Fig. 7 A, t = 0), the cultures were shifted to 37°C to see if Pds1 levels
would rise in either the cdc20-3 or hct1Δ strain, as an indication that Pds1 had become more stable. Pds1 levels rose in the cdc20-3 strain, but not in the hct1Δ strain, showing that the stability of Pds1 in anaphase is controlled by the Cdc20- rather than the Hct1-dependent APC (Fig. 7 A).

We next examined the half life of Pds1 during an anaphase arrest caused by the cdc15-2 mutation. Pds1 is unstable during this arrest and Cdc20 is required to exit from the arrest (Jaspersen et al., 1998; Tinker-Kulberg and Morgan, 1999). cdc15-2, cdc15-2 CDC28-VF, and cdc15-2 clb2Δ cells were arrested in anaphase, an epitope-tagged PDS1 gene driven by the GAL1 promoter was induced by adding galactose for 1 h, and its expression was terminated by adding glucose. The half life of Pds1 in cdc15-2 cells in this experiment was <15 min, but was >1.5 h in CDC28-VF cdc15-2 and clb2Δ cdc15-2 cells (Fig. 7 B), showing that the CDC28-VF and clb2Δ mutations compromise Cdc20-dependent APC activity.

Earlier studies argued that Hct1 and Cdc20 were specific for the A PC, with Cdc20 directing the ubiquitination of Pds1 and Hct1 directing that of mitotic cyclins (Visintin et al., 1997). The instability of Pds1 in G1 cells, which lack detectable Cdc20 (Prinz et al., 1998), prompted us to reexamine this issue. Wild-type, CDC28-VF, and cdc20-3 cells were arrested in G1 by alpha factor, or in the case of hct1Δ, which is resistant to alpha factor (Schwab et al., 1997), by the cdc28-13 mutation (Reed, 1980). Once arrested, wild-type, CDC28-VF, and cdc20-3 cells were shifted to 37°C (for cdc28-13 and cdc28-13 hct1Δ, asynchronous cultures were transferred to 37°C), pGAL-PDS1 was induced by adding galactose for two hours and then expression was shut off by adding glucose. Pds1 was equally unstable in wild-type, CDC28-VF, cdc20-3, and cdc28-13 cells, but was completely stable in the hct1Δ cdc28-13 cells. This control shows that the defect in Pds1 stability in CDC28-VF is specific to anaphase and, together with Fig. 7 A, shows that Pds1 is targeted for destruction by the Cdc20-dependent APC in mitosis and by the Hct1-dependent APC in G1. In addition, this experiment reinforces the conclusion derived from in vitro experiments in Fig. 6 that CDC28-VF has no defects in the Hct1-dependent APC activity in G1. Our results differ from those of Visintin et al. (1997), who found that stability of Pds1 in G1 is regulated by Cdc20. Their results may have been due to incomplete alpha factor arrest of the cdc20-1 allele used in their study. Our results agree with the recent observation that Clb2 is targeted for destruction by both the Cdc20- and Hct1-dependent forms of the APC (Yeong et al., 2000).

The stabilization of Pds1 in mitotic CDC28-VF cdc15-2 cells suggests that CDC28-VF is defective in the Cdc20-dependent APC. As a first step in investigating the biochemical defect of CDC28-VF, we examined the interaction between Cdc20 and the APC in anaphase, a time when the Cdc20-dependent APC is active. We arrested cdc15-2 and cdc15-2 CDC28-VF cells in anaphase, immunoprecipitated the APC with anti-Cdc26 antibodies, and examined the amount of associated Cdc20. Equal amounts of Cdc20 were immunoprecipitated from all three strains, but in CDC28-VF cells there was less associated Cdc20 (Fig. 8), even though the total level of Cdc20 was similar in wild-type and CDC28-VF.

Discussion

Cdc28 Activates the APC

We have shown that the CDC28-VF mutant is defective activating the Cdc20-dependent APC. CDC28-VF cells show a short delay in exiting mitosis, but this delay be-
comes more severe when the APC is compromised by spindle checkpoint activation or defects in the APC or its regulators. These phenotypes allowed us to show a requirement for Cdc28 in exiting mitosis. The defect in the exit from mitosis in CDC28-VF is correlated with reduced binding of Cdc20 to the APC and lower Cdc20-dependent APC activity. CDC28-VF cells have normal Hct1-dependent APC activity in G1, suggesting that their failure to
exit mitosis is the result of interrupting the chain of events that normally leads from mitosis to G1: reduced Cdc20-dependent APC activation prevents the activation of Cdc14, which normally leads to activation of Hct1 and increased levels of Sic1, the key steps in inactivating the mitotic activity of Cdc28 (Lim et al., 1998; Visintin et al., 1998; Jaspersen et al., 1999; Shirayama et al., 1999). Previously, it has been shown that Cdc28 inhibits exit from mitosis. High levels of Cdc28-associated kinase activity cause arrest in anaphase (Holloway et al., 1993; Surana et al., 1993), and inhibiting Cdc28 in mitotically arrested cells activates the APC, driving them through cytokinesis and into G1 (Amon, 1997; Li and Cai, 1997). These observations led to the conclusion that the major role for mitotic Cdc28 in regulating the APC was to inhibit the activity that drove cells out of mitosis. Our results show that Cdc28 has different effects on APC activity at different stages of mitosis. Cdc28/Cib activity activates the Cdc20-dependent APC to induce the metaphase to anaphase transition, but inhibits the Hct1-dependent APC, thus inhibiting cytokinesis and the transition from anaphase to G1. This pattern of regulation helps ensure chromosome segregation precedes cell division and allows budding yeast to regulate these events separately, a useful feature in an organism that specifies the site of cytokinesis long before spindle assembly.

Cdc28 Is Required for Recovery from Checkpoint-dependent Arrest

Proteins that were identified as members of the spindle checkpoint have two roles in keeping cells with depolymerized microtubules from leaving mitosis. Six of them, Mps1, Mad1-Mad3, Bub1, and Bub3, detect kinetochore-mitotic attachment defects that are not attached to the spindle (Hoyt et al., 1991; Li and Murray, 1991; Weiss and Winey, 1996) and prevent sister chromatid separation by inhibiting the Cdc20-dependent APC (Hwang et al., 1998; Kim et al., 1998). In contrast, Bub2 also identified as part of the spindle checkpoint (Hoyt et al., 1991), detects an unknown lesion and arrests cells in anaphase, probably by preventing the activation of Cdc14 (Alexandru et al., 1999; Fesquet et al., 1999; Fraschini et al., 1999; Li, 1999). The opposition between the inhibition of the Cdc20-dependent APC by the spindle checkpoint and activation by Cdc28 explains why CDC28-VF cells, which are defective in activating the Cdc20-dependent APC, have difficulty overcoming a checkpoint-dependent arrest.

We do not know how wild-type cells eventually escape from mitosis despite continued Mps1 overexpression, a process that could be described as adaptation to the spindle checkpoint (Rieder and Palazzo, 1992; Minn et al., 1996; Rudner and Murray, 1996). In particular, we cannot distinguish between constitutive mechanisms, such as stabilization of mitotic cyclins leading to a slow rise in Cdc28/Cib activity that eventually leads to APC activation, and induced mechanisms, such as a slow process initiated by components of the checkpoint that leads to reduced inhibition of Cdc20. Although we initially thought of CDC28-VF as an adaptation mutant (Minshull et al., 1996), we now believe that it achieves its effect by reducing the ability of Cdc28 to activate the Cdc20-dependent APC, whether or not the checkpoint is active. It is only when the checkpoint is active, however, that this reduction is sufficient to keep cells from leaving mitosis. This discussion highlights a general difficulty in studying adaptation mutants: the ability to overcome prolonged checkpoint-dependent arrests appears to be sensitive to small, checkpoint-independent defects in basic cell cycle machinery (Toczyski et al., 1997).

Other Mutants Share Phenotypes with CDC28-VF

Genetic analysis of Cdc28 has revealed two types of mutants, those that primarily affect G1 (cdc28-4, cdc28-13; Reed, 1980) and those that primarily affect exit from mitosis (CDC28-VF, cdc28-1N; Piggott et al., 1982; Surana et al., 1991). The mitotic mutants share phenotypes with clb2A and cks1-38. Do these four mutants, CDC28-VF, cdc28-1N, clb2A and cks1-38, have a common biochemical defect? Clb2A cells contain no Clb2, and therefore have reduced levels of mitotic Cdc28 activity (Grandin and Reed, 1993). The defects of cdc28-1N and cks1-38, which arrest cells in mitosis, may have more to do with altering the substrate specificity of Cdc28. When assayed by immunoprecipitation of Clb2-associated kinase, cdc28-1N strains have similar kinase activity to wild-type, but they have no kinase activity associated with Cks1-coupled beads (Surana et al., 1993; Kaisser et al., 1999). These results suggest that the primary defect in cdc28-1N is its failure to bind Cks1, which has been shown in frog and clam extracts to be essential for exit from mitosis and APC phosphorylation by Cdc2/Cyclin B (Patra and Dunphy, 1996; 1998; Shteinberg and Hershko, 1999).

Our only clue to the biochemical defect of Cdc28-VF is that it appears to have a small reduction in its specific activity (Fig. 4 D). We think this defect may be important because CD28-F88G, a mutant in the ATP binding site of Cdc28, also has reduced specific activity (Bishop et al.,...
2000), and shares phenotypes with CDC28-VF (Fig. 4 C). The cdc28-1N and cks1-38 mutations may produce their effects by reducing the level of the Cdc28/Cib/Cks1 complex, whereas in cib2Δ cells, the complexes of Cdc28 with the remaining Cib proteins may be less capable of activating the Cdc20-dependent A PC.

Although the Cdc28 activity is lower in CDC28-VF cells, we do not think that the defect in CDC28-VF is simply due to lower total Cdc28 activity per cell. Overexpression of Cib2 and Cib3, which raises Cdc28 activity in cells (Stueland et al., 1993), does not suppress the mitotic defect of CDC28-VF (data not shown). This result is consistent with the idea that the specific activity per Cdc28 molecule, not the total Cdc28 activity per cell, is critical for activating the exit from mitosis. An alternative explanation is that the different mutants change the substrate specificity of Cdc28, preventing phosphorylation of important mitotic substrates.

**A Cautionary Tale**

Protein phosphorylation is a common way of regulating protein activity. Mutating putative phosphorylation sites to nonphosphorylatable residues is a widely used technique for assessing the biological function of phosphorylation of specific proteins (Li et al., 1995; Zachariae et al., 1998; Jaspersen et al., 1999). Our analysis of CDC28-VF shows that such mutations can have unanticipated effects that are independent of phosphorylation.

It is difficult to tell how common such effects are. Our observations of CDC28-VF suggest that the studies conducted with the CDC28-AF, CDC28-VF, and CDC28-F mutants in budding yeast (as well as experiments with the corresponding mutants in Cdc2) should be reexamined to exclude the possibility that the observed effects of these mutants were due to phosphorylation-independent defects. A simple control is to ask whether the phenotype of the CDC28 mutants is exactly mimicked by deletion of SWE1. If so, the conclusions of the original experiments are secure. If not, phosphorylation-independent effects due to mutating the inhibitory residues may contribute to the observed phenotypes. In the general case, the ideal control is to show that inactivating the kinase that phosphorylates a particular protein produces a similar effect on the substrate’s activity as do the phosphorylation site mutants.

**Does Cdc28 Phosphorylate the APC?**

How does Cdc28 promote anaphase? Experiments in frogs, clams, and mammalian cell culture have all suggested that phosphorylation activates the A PC by modifying four of its subunits: Cdc16, Cdc23, Cdc27, and A pc1 (Bimé; Hershko et al., 1994; Peters et al., 1996; K otani et al., 1998, 1999; Patra and Dunphy, 1998). These proteins are phosphorylated during mitosis and the phosphorylated A PC has greater Cdc20-dependent activity in vitro, whereas dephosphorylation of purified A PC causes a loss of activity (Lahav-Barat et al., 1995; Shteinberg et al., 1999). Studies in clams have suggested that this phosphorylation is required for proper Cdc20 binding (Shteinberg et al., 1999). In the accompanying paper (Rudner and Murray, 2000, this issue), we show that the A PC is phosphorylated by Cdc28 in budding yeast, and that a defect in this phosphorylation causes reduced Cdc20-dependent A PC activity and contributes to the CDC28-VF phenotype.

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