Cdc28 Activates Exit from Mitosis in Budding Yeast

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Abstract. The activity of the cyclin-dependent kinase 1 (Cdk1), Cdc28, inhibits the transition from anaphase to G1 in budding yeast. CDC28-T18V, Y19F (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 Hct1-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 induce the metaphase to anaphase transition and initiate the transition from anaphase to G1 in budding yeast.

Key words: anaphase-promoting complex • Hct1 • 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), 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 (APC) or cyclosome, a multiprotein complex that is required for the ubiquitination of cyclin and other unstable substrates (King et al., 1995; Sudakin et al., 1995; Zachariae and Nasmyth, 1996, 1999; Yanagida et al., 1999). The activity of the APC 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 Hct1 (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). Cdc20-dependent A PC activity is inhibited by the spindle checkpoint, which senses defects in chromosome attachment to the spindle and delays the onset of anaphase until these defects are corrected (Rieder et al., 1995; Fang et al., 1998a; Hardwick, 1998; H wang et al., 1998; K im et al., 1998).

The second WD-40 protein, Hct1, also activates the A PC (Schwab et al., 1997; Sigrist and Lehner, 1997; Visintin et al., 1997; Kramer et al., 1998). In budding yeast, Hct1-dependent A PC activity is necessary for the ubiquitination and degradation of Clb2, the major mitotic cyclin, which causes the sudden drop in the protein kinase activity of Cdc28 at the end of mitosis and keeps the A PC active throughout G1 (Zachariae et al., 1998; Jas persen et al., 1999). The activation of the Hct1-dependent A PC depends on the prior activation of the Cdc20/A PC, and this dependency helps ensure that the events of mitosis occur in the proper sequence (Lim et al., 1998; Visintin et al., 1998; Shirayama et al., 1999; Y eong et al., 2000).

In budding yeast, active Cdc28 inhibits the transition from anaphase to G1 (A mon, 1997; Li and Cai, 1997). One of the primary targets that Cdc28/Clb2 complexes inhibit is Hct1. Phosphorylation of Hct1 by Cdc28/Clb complexes prevents it from binding to and activating the A PC (Zachariae et al., 1998; Jaspersen et al., 1999). This inhibition is opposed by the phosphatase Cdc14, which de-phosphorylates Hct1, initiating a positive feedback loop that drives the cell into G1 (Visintin et al., 1998; Jaspersen et al., 1999). A s Hct1 activity rises, the rate of Clb destruction increases, reducing the kinase activity of Cdc28 and further activating Hct1. The CDK inhibitor, Sic1 (Mendenhall, 1993; Donovan et al., 1994), which inhibits Cdc28/Clb
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; Visentin 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; Hershko et al., 1994; M inshull et al., 1994; Shteinberg 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). This phosphorylation is correlated with activating the A PC and binding of Fizzy, the Cdc20 homologue in frogs (Peters et al., 1996; F ang et al., 1998b; K otani et al., 1998; Shteinberg et al., 1999). In embryonic cell cycles, no Hct1 homologue is present (Sigrist and Lehner, 1997; Lorca 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 Hct1 or Sic1 is required for cells to exit from mitosis (Schwab et al., 1997; Visentin et al., 1997). Since both Hct1 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 18 (Amon et al., 1992; Sorger and Murray, 1992; Booher et al., 1993). Tyrosine 19 is phosphorylated by Swel (the homologue of Wee1 in fission yeast) and dephosphorylated by Mih1 (the homologue of Cdc25 in fission yeast; Russell et al., 1989; Booher et al., 1993). Phosphorylation on the homologous sites of fission yeast and vertebrate Cdc2 controls the timing of entry into mitosis and also can be induced by the checkpoints that detect unreplicated or damaged DNA (Gould and Nurse, 1989; Enoch and Nurse, 1990; Norbury et al., 1991; Jin et al., 1996; Rhind et al., 1997). In budding yeast, tyrosine 19 is phosphorylated during S-phase, but Cdc28-T18A, Y19F (CDC28-AF-HA) cells respond normally to the DNA damage checkpoint (A mon et al., 1992; Sorger and M urray, 1992). 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).

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; Healy et al., 1991), are spindle checkpoint defective and have increased phosphorylation on tyrosine 19 of Cdc28 (M inshull et al., 1996; W ang and B urke, 1997). The premature exit from mitosis in cdc55Δ cells with damaged spindles is suppressed by Cdc28-T18V, Y19F (CDC28-VF), suggesting that inhibitory phosphorylation of Cdc28 might work in concert with Hct1 and Sic1 to reduce Cdc28-associated activity at the end of mitosis.

Here, we show that this hypothesis is incorrect. Although CDC28-VF has defects in leaving mitosis, these are not due to a lack of inhibitory phosphorylation, but reflect a second defect of CDC28-VF. This defect slows the normal activation of the Cdc20-dependent A PC and reveals that Cdc28-associated activity is essential for the activation of the A PC in mitosis.

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; R odney R othstein, Columbia University, N Y). Standard genetic techniques were used to manipulate yeast strains (Sherman, 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 TG1 and DH5α 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 DR 1541 (CDC28-AF), A DR 2035 (CDC28-F86G), K H 208 (sweΔA), A DR 484 (cdc28-1N), A DR 840 (cdc4), A DR 314 (cib2A), A DR 719 (cib2-31), A DR 1147 (cibc1-1), L H 226 (doc1-1), L H 125 (cdc28-1), A DR 1435 (hct1Δ), J C 126 (cdc5-1), A DR 1298 (cibc1-3), and K 193 (cibc1-5). For all crosses, at least 22 tetrads were analyzed.

BAR1 was deleted using p G 51 (a gift of J eff U bersax, U niversity of California, Berkeley, CA). pDS1-myc18 strains were made using a PDS1-myc18 replacement plasmid (Shirayama et al., 1998). pCUP-GFP12-lacI, lacO:TRP1 and lacO:LEU2 were integrated using pSB116 (Biggins et al., 1999), pA FS52, and pA FS59 (Strait et al., 1996), respectively. pGAL-MPS1 strains were made with pA FS120 (Hardwick et al., 1996). M AD 3 was deleted using pKH181 (Hardwick et al., 2000). sweΔ strains were made by crossing J M 449 (a gift of J eff M inshull), M axygen, Redwood City, CA) to the appropriate strains. M H11 was deleted using pIP33 (a gift of Peter Sorger, Massachusetts Institute of Technology, Cambridge, MA). CDC55 was deleted using pM 6 (M inshull et al., 1996). cdc28-1 strains were made with cdc28-1N (Surana et al., 1991) to the appropriate strains. CDC28-F86G strains were made by crossing JA U 02 (a gift of J eff U bersax, U niversity of California, San Francisco, CA) to the appropriate strains. The 2μ-CDC28 plasmid is EF190 (a gift of E ric F oss, Fred Hutchinson Cancer Research Institute, Seattle, WA) and is the CDC28 gene cloned into pRS425 (Sikorski and H ieter, 1989). pHS3-GFF-TUB1 was integrated using pA FS71 (a gift of A aron S trong, Har vard Medical School, Boston, MA) to the appropriate strains. The CDC28 gene was then cloned into K 8029 (Shirayama et al., 1998) to the appropriate strains. pGAL-PDS1-HA strains were made by crossing R TK 43 (J aspern et al., 1998) to the appropriate strains.

Mutants in CDC28-VF-HA, CDC28-F-HA, CDC28-V-HA, and CDC28-HA were made as described previously (Booher et al., 1993). CDC28-AF-HA was made by cloning the HindIII fragment from pSF35 (Peter Sorger, MIT, Cambridge, MA) into pDR96 (a gift of R ay Dea shales, California Institute of Technology, Pasadena, CA) cut with HindIII/Xhol to create pA R 155, which was used to create a CDC28-AF HA strain as previously described for pDR96 (Booher et al., 1993). The pGAL-CDC28 plasmids were made as follows: full-length CDC28-HA (pA RG263) was made by cloning the 1.3-kb HindIII HindII fragment from CDC28-VF-HA into pDR96 and replacing it with the same fragment from pDR47. The B SphI-BamHI fragment of pDR96 or pA R 106 was then cloned into pDK20 (a gift of D oug K elloge, U niversity of California, Santa Cruz, CA) cut with SmaI and BamHI, to create pA R 109 (ypLp-GAL-CDC28URA3) and pA R 108 (ypLp-GAL-CDC28-VF-URA3). The plasmids were cut with StuI and integrated at the URA3 locus.

pGAL-CLB2::L176 strains were made with pAR39. An A EOR1/BamHI fragment of CB2 that lacks the first 176 amino acids was amplified by PCR and cloned into pDK20 cut with EcoRI and BamHI to create pAR39. The plasmid was cut with StuI for integration at the URA3 locus.

csk1-38 strains are csk1-38::KAN1 covered by csk1-38 integrated at TRP1 (pB 1A 8). C k51 was deleted by the PCR-targeting method. Diploid cells were transformed with a casette containing the bacterial KAN1 gene, which confers G418 resistance in W303. The cassette was amplified by PCR from pFA6a-kanMX6 (Longtine et al., 1998) with primers that contain the sequences that flank the CAS1 open reading frame. pAR183 (cksk1-38::TRP1) was constructed by cloning an BamHI/SphI fragment from pSE1 (T ang and R eer, 1993) into YIP204 (G ietz and Sugin, 1988), the resulting plasmid was cut with EcoRV and inserted into the K S K1/csk1-3::KAN1 heterozygote at the TRP1 locus. The diploid was sporulated, and a resulting spore, A DR 1767, which is temperature sensitive, TRP1, and KAN1 was used to create the strains used in this study.

H CT1 was deleted using pA R 127. An A EOR1/HindI11 fragment of the H 1 locus was amplified by PCR and cloned into pSK(-) (Stratagene) to create pAR125. An XbaI/Smal fragment of the H1S3 gene was then

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Table I. Strain List

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*All strains are isogenic to W303-1a (MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trpl-1 ura3-1).

1All pGAL-MPS1 strains are derived from crosses with KH153.

cloned into pA R125 cut with SphI/XmnI, to create pA R 127, which replaces the entire HTC1 open reading frame with HIS3. The EcoRI/NcoI fragment of pA R 127 was used to transform yeast.

CDC20 was tagged at the N/H2 terminus. A cassette containing 12 myc tags (pL4 H71) was inserted into pCM4 (Hwang et al., 1998) cut with BstEII, to create pL4 H83. The resultant plasmid was cut with EcoRI and BglII, removing the CEN A-R S sequences, blunted, and religated to create pL4 H92. This plasmid was cut with BspE1 for integration at the CDC20 lo-

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Table II. Genetic Interactions of CDC28-VF

| cdc23-1 | sl | sl | sl | sl | + | + | + | sl | sl | sl | + |
| cdc16-1 | sl | + | + | + | + | + | + | sl | ND | ND | + |
| doc1-1  | sl | sl | sl | sl | sl | sl | sl | sl | sl | sl | sl |
| cdc20-1 | sl | sl | sl | sl | sl | sl | sl | sl | sl | sl | sl |
| hclΔ   | sl | sl | sl | sl | sl | sl | sl | sl | sl | sl | sl |
| cdc5-1  | sl | sl | sl | sl | sl | sl | sl | sl | sl | sl | sl |
| cdc13-1 | + | + | + | + | + | + | + | + | + | + | + |
| cdc15-2 | + | + | + | + | + | + | + | + | + | + | + |

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 LHH371. 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); 10 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 peptone (YPEP) and peptone (YPEP) + 2% raffinose, spotted onto a YEP + 2% galactose plate, and unpruned 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 counted.

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 lacO repeat to bounds of lacZ, 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 scored live, or fixed for 10 min in 3.7% 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 anaphase arrest of pGA1-CL C. Reactions were stopped by adding 15 μl of 2× SDS sample buffer and heating samples to 99°C. Samples were run on a 15% polyacrylamide gel, stained, and dried. Kinase reactions were performed in 15 μl of 2× kinase buffer (80 mM NaPO4, 15 mM MgCl2, 2 mM EGTA). All washes were performed on ice. Kinase reactions were performed in 15 μl of kinase buffer containing 1 μM DTT, 25 μM ATP, 25 μg histone H1 (Upstate Biotechnology) and 1 μCi 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× SDS sample buffer and heating samples to 99°C for 5 min. Samples were run on a 13% polyacrylamide gel, stained, and dried. Kinase gels were quantified using a Molecular Dynamics PhosphoImager and ImageQuant software.

Histone H1 Kinase Assays

For histone H1 kinase reactions, 0.33 μg of anti-Cib2 or anti-Cib3 antibody was used for immunoprecipitation in 1× mg of cell lysate. A ffer immunoprecipitation, the beads were 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 twice in kinase buffer (80 mM NaCl, 50 mM Tris-Cl, pH 7.4, 50 mM NaF, 5 mM Na3PO4, 1 mM Na3VO4, 1 mM EGTA, 2 mM EDTA), 0.1% Triton X-100, 1 mM Na3VO4, 1 mM PM SF, and leupeptin, pepstatin and chymostatin all at 1 μg/ml and an excess of acid washed glass beads for 2 pulses of 90 s, incubating on ice for 5 min between each pulse. The resulting lysate was separated from the glass beads and centrifuged in a microfuge at 14,000 rpm for 5 min to remove insoluble material. Protein concentration of each lysate was determined using Bradford reagent (0.04% Coomassie blue G-250 dissolved in 4.75% ethanol, then mixed with 8.5% o-phosphoric acid and H2O) and samples were normalized based on these measurements. A portion of the lysate was mixed with an equal volume of 2× SDS sample buffer. Standard methods were used for PAGE and protein transfer to nitrocellulose (Schleicher and Schuell; 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 fter washing in PBST (PBS + 0.1% Tween-20; Mannatis et al., 1982), the blots were then incubated in HR P-conjugated anti-rabbit or anti-mouse antibodies (A mersham Pharmacia Biotech) at a 15,000 dilution in PBST for 30 min at 25°C, washed again, incubated in ECL detection reagents (A mersham Pharmacia Biotech) or Renaissance reagents (NEN Life Science Products), using the manufacturer’s instructions, and then exposed to X-Omat film (Kodak).

The following antibodies were used in Western blots: 9E10 ascites (BabCO) was used at a dilution of 1:10,000 in PBST + 0.02% NaN3 after blocking in 4% nonfat dried milk in PBST. Afinity-purified rabbit polyclonal anti-Cib2 and anti-Cib3 antibodies (Kellogg and Murray, 1995) were used at a dilution of 1:1,000 in blocking buffer (2% BSA, PBST, 0.5% NaCl, 0.02% NaN3). Rabbit polyclonal anti-Sic1 serum (a gift of Mike M endenhall, University of Kentucky, Lexington, KY) was used at 1:1,000 in blocking buffer (4% nonfat dried milk, 2% BSA, PBST, 0.02% NaN3) + 10 μg/ml cell lysate made from sic1Δ cells (J M 408, made with MDM p203; a gift of Mike M endenhall). 12CA 5 ascites (BabCO) was used at 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 protein A CL-4B Sepharose beads (Sigma-Aldrich) that had been equilibrated in lysate buffer. The beads were rotated at 4°C for 1–2 h. The beads were manipulated as described below.

Phosphotyrosine Detection

For antiphosphotyrosine blots, 2 μg of 12CA 5 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.

The APC assay was conducted as previously described (Hardwick et al., 1999). 25 nM of purified Hct1 fragment conjugated to HRP, RC20H (Transduction Laboratories), di- 

The beads were washed three times 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 low salt kinase buffer (10 mM NaCl buffer (200 mM NaCl), 20 mM Hepes-KOH, pH 7.4, 5 mM MgCl2). The immunoprecipitate 10–20 mg of cell lysate. After immunoprecipitation, the beads were washed three times in Cdc20 bead buffer (200 mM NaCl, 50 mM Tris-Cl, pH 7.4, 50 mM NaF, 5 mM EDTA, 5 mM EGTA, 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 nM of purified Hct1 (J. aspersen et al., 1999) was added to some samples during the ubiquitina-

Cdc20 Binding to the APC

For Cdc20 binding experiments, 3 μg of anti-Cdc26 antibody was used to immunoprecipitate 10–20 mg of cell lysate. After immunoprecipitation, the beads were washed three times in Cdc20 bead buffer (200 mM NaCl, 50 mM Tris-Cl, pH 7.4, 50 mM NaF, 5 mM EDTA, 5 mM EGTA, 0.1% NP-40, 1 mM DTT), and then twice in low salt kinase buffer (10 mM NaCl, 20 mM Hepes-KOH, pH 7.4, 5 mM MgCl2). The immunoprecipi-

Results

CDC28-VF Impairs Mitotic Exit

Since CDC28-VF suppresses the checkpoint defect of cdc55A (Minsell 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 CDK inhibitor, Sic1 (Schwob et al., 1994), disappears at the same time (30 min) in wild-type and CDC28-VF.

Athough 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 parado
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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 behave identically to CDC28-VF. However, CDC28-AF
behave 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; Hadwiger 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/ CDC28 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-
nation with mutations in components of the A PC (cdc23-1, cdc16-1, apc10-1, formerly doc1-1; Lamb et al., 1994; Irniger et al., 1995; Hwang and Murray, 1997) and positive regulators of the A PC (cdc5-1, hct1Δ and cdc20-1; Schwab et al., 1997; Visintin et al., 1997; Charles et al., 1998; Shirayama et al., 1998). These double mutants are inviable at all temperatures, and although the double mutant spores are able to germinate, they die in microcolonies of large-budded cells (data not shown), indicating a terminal arrest in mitosis. These genetic interactions are specific to the A PC and regulators of the A PC, because two other mutants that arrest in mitosis, cdc13-1 (which activates the DNA damage checkpoint) and cdc15-2 (which arrests cells in anaphase) are both viable in combination with CDC28-VF.

The interactions of CDC28 alleles and clb2Δ with Mps1 overexpression and the cdc23-1 mutant are correlated with each other: cdc28-1N, CDC28-F88G, and clb2Δ are synthetically lethal in combination with cdc23-1 and cannot proliferate when overexpressing Mps1, whereas cdc28-4 is viable in combination with cdc23-1 or Mps1 overexpression (Table II; also, see Irniger et al., 1995). CDC28-F and CDC28-AF, which have milder phenotypes than CDC28-VF, are synthetically lethal with cdc23-1, but viable in combination with cdc16-1, a weaker A PC mutant (Table II). Lastly, swe1Δ is viable in combination with cdc23-1 and cdc16-1 (Table II), demonstrating that Swe1 is unlikely to have a role in promoting the exit from mitosis.

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 apcΔ double mutant, since it is impossible to create such a mutant. We overcome 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 rearrest 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 mad2Δ 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 A PC, which triggers anaphase, and the Hct1-dependent A PC, 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 (K H181), cdc28-1N (A D R1899), cdc28-4 (A D R1901), clb2Δ (A D R1606), and cks1-38 (A D R1903) 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 (A D R477) and CDC28-VF (A D R509) 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 (K H181), and CDC28-F88G (A D R2034) 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 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 overexpressing 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. 7A).

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 (Aspensen 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. 7B), showing that the CDC28-VF and clb2Δ mutations compromise Cdc20-dependent APC activity.

Earlier studies argued that Hct1 and Cdc20 were specific factors 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 (Schrab 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. 7A, 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 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 Cln2 is targeted for destruction by both the Cdc20- and Hct1-dependent forms of the A PC (Yoon et al., 2000).

The stabilization of Pds1 in mitotic CDC28-VF cdc15-2 cells suggests that CDC28-VF is defective in the Cdc20-dependent A PC. As a first step in investigating the biochemical defect of CDC28-VF, we examined the interaction between Cdc20 and the A PC in anaphase, a time when the Cdc20-dependent A PC is active. We arrested cdc15-2 and cdc15-2 CDC28-VF cells in anaphase, immunoprecipitated the A PC with anti-Cdc26 antibodies, and examined the amount of associated Cdc20. Equal amounts of A PC was 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 cells.

**Discussion**

**Cdc28 Activates the APC**

We have shown that the CDC28-VF mutant is defective activating the Cdc20-dependent A PC. CDC28-VF cells show a short delay in exiting mitosis, but this delay be-
comes more severe when the A PC is compromised by spindle checkpoint activation or defects in the A PC 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 A PC and lower Cdc20-dependent A PC activity. CDC28-VF cells have normal Hct1-dependent A PC 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 A PC 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 (Li et al., 1993; Kaiser et al., 1999). These results suggest that Cdc28, 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; Fraschini et al., 1999; Li, 1999). The opposition between the inhibition of the Cdc20-dependent A PC by the spindle checkpoint and activation by Cdc28 explains why CD C28-VF cells, which are defective in activating the Cdc20-dependent A PC, 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; Min et al., 1996; Rieder and Murray, 1996). In particular, we cannot distinguish between constitutive mechanisms, such as stabilization of mitotic cyclins leading to a slow rise in Cdc28/Cby activity that eventually leads to A PC 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 CD C28-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 A PC, 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 CD C28 has revealed two types of mutants, those that primarily affect G1 (cd28-4, cd28-13; Reed, 1980) and those that primarily affect exit from mitosis (CD C28-VF, cd28-1N; Piggott et al., 1982; Surana et al., 1991). The mitotic mutants share phenotypes with clb2a and cks1-38. Do these four mutants, CD C28-VF, cd28-1N, 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 cd28-1N 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, cd28-1N strains have similar kinase activity to wild-type, but they have no kinase activity associated with Cks1-coupled beads (Surana et al., 1991; Kaiser et al., 1999). These results suggest that the primary defect in cd28-1N is its failure to bind Cks1, which has been shown in frog and clam extracts to be essential for exit from mitosis and A PC phosphorylation by Cdc2/Cyclin B (Patra and Unphy, 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 CD C28-F88G, a mutant in the ATP binding site of Cdc28, also has reduced specific activity (Bishop et al., 1996; Rudner et al., 1997). 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; Fraschini et al., 1999; Fraschini et al., 1999; Li, 1999). The opposition between the inhibition of the Cdc20-dependent A PC by the spindle checkpoint and activation by Cdc28 explains why CD C28-VF cells, which are defective in activating the Cdc20-dependent A PC, 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; Min et al., 1996; Rieder and Murray, 1996). In particular, we cannot distinguish between constitutive mechanisms, such as stabilization of mitotic cyclins leading to a slow rise in Cdc28/Cby activity that eventually leads to A PC 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 CD C28-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 A PC, 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).
substrates. The different mutants change the substrate specificity inactivating the exit from mitosis. An alternative explanation is the idea that the specific activity per Cdc28 molecule, as do the phosphorylation site mutants.

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 defects 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 p1c (Bimé; Hershko et al., 1994; Peters et al., 1996; K otani et al., 1998, 1999; Patra and D unphy, 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-Baratz 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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S. cerevisiae


