Cdc28 activates exit from mitosis in budding yeast

Citation for published version:
Rudner, AD, Hardwick, KG & Murray, AW 2000, 'Cdc28 activates exit from mitosis in budding yeast' The Journal of Cell Biology, vol. 149, no. 7, pp. 1361-76. DOI: 10.1083/jcb.149.7.1361

Digital Object Identifier (DOI):
10.1083/jcb.149.7.1361

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
The Journal of Cell Biology

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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 (A PC), a defect shared with other mutants that lower Cdc28/Clb activity in mitosis. CDC28-VF has reduced Cdc20-dependent APC activity in mitosis, but normal Hct1-dependent APC activity in the G1 phase of the cell cycle. The defect in Cdc20-dependent APC 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 cyclin-dependent kinase 1 (Cdk1), known as Cdc28 in budding yeast and Cdc2 in other eukaryotes, 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 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 (R ieder et al., 1995; F ang et al., 1998a; H ardwick, 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; V isintin 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 (Z achariae et al., 1998; Jaspersen 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 (L im et al., 1998; V isintin 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; L i and C ai, 1997). One of the primary targets that Cdc28/Clb2 complexes inhibit is H ct1. Phosphorylation of H ct1 by Cdc28/Clb complexes prevents it from binding to and activating the A PC (Z achariae 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 (V isintin 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; D onovan et al., 1994), which inhibits Cdc28/Clb

Abbreviations used in this paper: A PC, anaphase-promoting complex; Cdk, cyclin-dependent kinase; DAPI, 4',6-diamidino-2-phenylindole; GFP, green fluorescent protein.

© The Rockefeller University Press, 0021-9525/2000/06/1361/16 $5.00
The Journal of Cell Biology, Volume 149, Number 7, June 26, 2000 1361–1376
http://www.jcb.org

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

Does Cdc28 also play a role in inducing anaphase? In frog and clam egg extracts, activation of the APC depends on active Cdc2/Cyclin B complexes (Felix et al., 1990; Hershko et al., 1994; Minshull et al., 1994; Shtenberg and Hershko, 1999). This activating role might be explained by the finding that Cdc2/Cyclin B can phosphorylate subunits of the APC (Patra and D’urphy, 1998; Kottani et al., 1999), and this phosphorylation is correlated with activating the APC and binding of Fizzy, the Cdc20 homologue in frogs (Peters et al., 1996; Fang et al., 1998b; Kottani et al., 1998; Shtenberg et al., 1999). In embryonic cell cycles, both Hct1 homologue is present (Sigrist and Lehner, 1997; Lorca et al., 1998), suggesting that Cdc20-dependent APC 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; Visintin et al., 1999). 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; Bohler 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; Russell et al., 1989; Bohler 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., 1993; Jin et al., 1996; Rind et al., 1997). In budding yeast, tyrosine 19 is phosphorylated during S-phase, but CDC28-T18A, Y19F (CDC28-AF) cells respond normally to the DNA damage checkpoint (Amon et al., 1992; Sorger and Murray, 1992). Inhibitory phosphorylation of Cdc28 is required for the bud emergence: or morphogenesis checkpoint, which delays mitosis in cells that have not budded (Lew and Reed, 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 (Minshull et al., 1996; Wang and Burke, 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 APC and reveals that Cdc28-associated activity is essential for the activation of the APC in mitosis.

Materials and Methods

Table I lists the strains used in this work. All strains are derivatives of the W303 strain background (W303-1a; Rody et al., Columbia University, New York, NY). 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 T1 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), ADR 1541 (CDC28-AF), ADR 2035 (CDC28-F88G), KH 208 (swelΔ), ADR 484 (cdc28-1N), ADR 840 (cdc28-4), ADR 314 (cb2A), ADR 719 (cdc23-1), ADR 1147 (cdc61-1), LH 226 (doc1-1), LH 125 (cdc28-1), ADR 1435 (hct1Δ), JC 126 (cdc5-1), ADR 1298 (cdc13-1), and K193 (cdc5-2). For all crosses, at least 22 tetrads were analyzed.

Bar1 was deleted using pGSt1 (a gift of Jeremy Thorner, University 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 (Birdsall et al., 1999), pα F52, and pα F59 (Strait et al., 1996), respectively. GA1-MPS1 strains were made with pα F5120 (Hardwick et al., 1996). MAD3 was deleted using pKH181 (Hardwick et al., 2000). swelΔ strains were made by crossing M 449 (a gift of Jeremy Minschull, Maxygen, Redwood City, CA) to the appropriate strains. M11H was deleted using pIp33 (a gift of Peter Sorger, Massachusetts Institute of Technology, Cambridge, MA). CDC28 was deleted using pM6 (Minschull et al., 1996). clb2 strains were made by crossing K1930 (Surana et al., 1991) to the appropriate strains. CDC28-F88G strains were made by crossing JAU02 (a gift of Jeff Ubersax, University of California, San Francisco, CA) to the appropriate strains. The 2μ-CDC28 plasmid is E190 (a gift of Eric Foss, Fred Hutchinson Cancer Research Institute, Seattle, WA) and is the CDC28 gene cloned into pRS425 (Sikorski and Hieter, 1989). pHis3-GFP-TUB1 was integrated using pα F571 (a gift of A Aron, Stanford, Harvard Medical School, Boston, MA). cdc20-3 strains were made by crossing K8209 (Shirayama et al., 1998) to the appropriate strains. pGAL-PDS1-HA strains were made by crossing RTK43 (Jaspersen et al., 1998) to the appropriate strains. Mutations in CDC28-VF-HA, CDC28-B-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 p5F35 (Peter Sorger, MIT, Cambridge, MA) into pRD96 (a gift of Ray Deshaies, California Institute of Technology, Pasadena, CA) cut with HindIII/XhoI to create pα R155, which was used to create a CDC28-AF-HA strain as previously described for pRD96 (Booher et al., 1993). The pGAL-CDC28 plasmids were made as follows: full-length CDC28-HA (pα F109), was made by cloning the 1.3-kb HindIII fragment of pRD96 into pGAL-CDC28-VF-HA from pRD96 and replacing it with the same fragment from pRD47. The BstBI-BamHI fragment of pRD96 or pα F106 was then cloned into pDK20 (a gift of Doug Kellogg, University of California, Santa Cruz, CA) cut with Sma and BamHI, to create pα R109 (pIP-GAL-CDC28-U3A) and pα R108 (pIP-GAL-CDC28-VF-U3A). The plasmids were cut with StuI and integrated at the URA3 locus.

pGAL-CLB2Δ176 strains were made with pα R39. An pEOR1/βamH1 fragment of CLB2 that lacks the first 176 amino acids was amplified by PCR and cloned into pDK20 cut with EcoR1 and βamH1 to create pα R39. The plasmid was cut with Stul for integration at the URA3 locus. cks1-38 strains are cks1Δ::KANm covered by cks1-38 integrated at TRP1 (pα R183). CK51 was deleted by the PCR-targeting method. Diploid cells were transformed with a cassette containing the bacterial KANm gene, which confers G418 resistance in W303. The cassette was amplified by PCR from pγa6a-kanMX6 (Longtine et al., 1998) with primers that contain the sequences that flank the CK51 open reading frame. pα R183 (cks1-38TRP) was constructed by cloning an BamHI/Sphi fragment from pS271 (Tang and Reed, 1993) into YIp1ac204 (Getz and Sugino, 1988), the resulting plasmid was cut with EcoRV and integrated into the K511/cks1Δ::KANm heterozygote at the TRP1 locus. The diploid was sporulated, and a resulting spore, ADR176, which is temperature sensitive, TRP1, and KANm was used to create the strains used in this study.

HCT1 was deleted using pα R127. An pEOR1/βamH1 fragment of the HCT1 locus was amplified by PCR and cloned into pSK (−) (Stratagene) to create pα R125. An pXbaI/Smal fragment of the H1S3 gene was then
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 trp1-1 ura3-1).

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

cloned into pA R125 cut with SpeI/XmnI, to create pA R127, which replaces the entire HCT1 open reading frame with HIS3. The EcoR1/NotI fragment of pA R127 was used to transform yeast.

CDC20 was tagged at the NH2 terminus. A cassette containing 12 myc tags (pLH 71) was inserted into pCM4 (Hwang et al., 1998) cut with BstEII, to create pLH 83. The resultant plasmid was cut with EcoNI and BglII, removing the CEN A-RS sequences, blunted, and religated to create pLH 92. This plasmid was cut with BspE1 for integration at the CDC20 lo-

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Table II. Genetic Interactions of CDC28-VF

<table>
<thead>
<tr>
<th>CDC28-VF</th>
<th>CDC28-V</th>
<th>CDC28-F</th>
<th>CDC28-AF</th>
<th>swe1Δ</th>
<th>CDC28-FAS8G</th>
<th>cdc28-1N</th>
<th>cbl2Δ</th>
<th>cdc28-4</th>
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sl, Synthetic lethal; ND, not determined; +, viable.

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 (YE P) + 2% raffinose, spotted onto a YEP + 2% galactose plate, and unbudded cells were picked out into a grid with a dissection needle. The number of cells in each microcolony was counted at different times after incubation at 30°C. Each bud was counted as a cell, and the original cells that did not bud were scored live, or fixed for 10 min in 3.7% paraformaldehyde at 4°C.

For serial dilution and spotting, cells were prepared in a multiwell dish or a microtiter plate, and using a multiprong appicator, ~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 to repeats of lacO, 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 pGAL-CLB2-A176 was visualized using pHIS3-GFP-TUB1. GFP-Tub1 was induced by transferring cells to complete synthetic medium (CSM)-His + 2% galactose + 10 mM 3-aminitolazolene for the first 30 min after alpha factor removal. Cells were then transferred into YEP + 2% galactose. Spindle length was scored in living cells by fluorescence microscopy. The anaphase arrest of cdc15-2 was scored by fixing cells for 5 min in 70% ethanol, washing in 50 mM Tris-Cl, pH 7.5, and then resuspending in 50 mM Tris-Cl, pH 7.4, + 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes). Cells were then mounted on slides and viewed by fluorescence microscopy.

Immunoprecipitation and Western Blots

In experiments where only Western blots are shown, yeast extracts were prepared by bead beating (multitube bead beater; Biospec) frozen cell pellets in 1X SD S sample buffer (2% SDS, 80 mM Tris-Cl, pH 6.8, 10% glycerol, 10 mM EDTA, 0.02% bromophenol blue, 1 mM NaVO₄, 1 mM PMSF, and leupeptin, pepstatin and chymostatin all at 1 µg/ml) and an excess of acid washed glass beads (Biospec) for 1 pulse of 90 s. Samples were then normalized based on OD_{600} readings of the original yeast samples taken during the time course. We have found that this method allows even loading of the samples and works as well as other techniques.

Yeast extracts for immunoprecipitation and Western blots were made by bead beating frozen cell pellets in lysis buffer (100 mM NaCl, 50 mM Tris-Cl, pH 7.5, 50 mM NaF, 50 mM Na-glycerophosphate, pH 7.4, 2 mM EGTA, 2 mM EDTA, 0.1% Triton X-100, 1 mM NaVO₄, 1 mM PMSF, 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 2X 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 filter 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 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 (NEI 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 1:1,000 in PBST + 0.2% NaN₃ after blocking in 4% nonfat dried milk in PBST. A affinity-purified rabbit polyclonal anti-Cib2 and anti-Cib3 antibodies (K ellogg and Murray, 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-Sic serum (a gift of M ike 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). 12CA5 ascites (Babco) was used at 1:1,000, rabbit polyclonal anti-Cdc16 and anti-Cdc23 (L amb et al., 1994) were used at 1:2,000, and anti-Cdc27 (Lamb et al., 1994) was used at 1:2,500. The 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 microtube 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 lysis buffer. The beads were rotated at 4°C for 1–2 h. The beads were manipulated as described below.

Histone H1 Kinase Assays

For histone H1 kinase reactions, 0.33 µg of anti-Cib2 or anti-Cib3 antibody was used for immunoprecipitation in 1–5 mg of cell lysate. A fter 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 Na-β-glycerophosphate, pH 7.4, 15 mM MgCl₂, 20 mM EGTA). All washes were performed on ice. Kinase reactions were performed in 15 µl of kinase buffer containing 1 mM DTT, 25 µM ATP, 2.5 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 2X 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.

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-
sate. The beads were washed three times in kinase bead buffer and twice in PBS. Standard methods were used for PAGE and protein transfer to nitrocellulose. The blot was then blocked in P-Tyr blocking buffer (1% BSA, 10 mM Tris-Cl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) and incubated overnight at 4°C in blocking buffer containing an anti-P-Tyr Fab fragment conjugated to HRP, RCOH (Transduction Laboratories), diluted to 1:2,500. The blot was washed five times in TBS (10 mM Tris-Cl, pH 7.5, 100 mM NaCl, 0.1% Tween 20), incubated in SuperSignal chemiluminescent substrate (Pierce Chemical Co.), using the manufacturer's instructions, and then exposed to X-ray film. A further exposure, the blot was stripped in an SD 5-containing buffer, and reprobed with the 12CA5 antibody to confirm that the same amount of Cdc28 had been immunoprecipitated.

APC Assay
For a APC assay, 1 μg of rabbit polyclonal anti-Cdc26 antibody (Hwang and Murray, 1997) was used to immunoprecipitate the A PC from 5 mg of cell lysate. The A PC 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 a P AC 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 nM of purified Hct1 (Rudner et al., 1998) was added to some samples during the ubiquitination reaction.

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. A fter 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 EGTA, 5 mM EDTA, 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 immunoprecipitates were then processed for Western blots as described above.

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 into G1 by the mating pheromone alpha factor and released into the cell cycle. Only cells had budded, alpha factor was readded to arrest cells that had completed the cycle. This regimen allows us to look closely 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.

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 Cib2, inactivate Cib2-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 Msps1, which arrests cells in mitosis by activating the checkpoint, but does not damage the spindle (Hardwick et al., 1996). After about eight hours, wild-type cells overcome the arrest, divide, and resume proliferating. In contrast, CDC28-VF cells overexpressing Msps1 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 mad3A mutation, which inactivates the spindle checkpoint (Li and Murray, 1991; Hardwick et al., 2000). mad3A 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. swe1Δ 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 threonine 18 in swe1Δ 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. mihΔ 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 mihΔ cells (Fig. 3 B and data not shown), and mihΔ 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
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; 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 2 μm 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 I). 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-VF, 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-VF and CDC28-A F, 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 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 reaest 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; Alexander 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 (KH153), CDC28-VF (KH181), cdc28-1N (ADR1899), cdc28-4 (ADR1901), clb2Δ (ADR1902), and cks1-38 (ADR1903) 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 (KH153), 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 spotted onto YEP + 2% glucose (left) or YEP + 2% galactose (right). The plates were incubated at 30°C for 2 d.

B, wild type  
CDC28-VF

HH1 phosphorylation

anti-Clb2 IP
anti-Clb3 IP

C, GAL-MPS1 plus glucose galactose

wild type
CDC28-VF

D, GAL-MPS1 plus glucose galactose

CDC28 + 2μ-TRP1
CDC28 + 2μ-CDC28
CDC28-VF + 2μ-TRP1
CDC28-VF + 2μ-CDC28

bated at 30°C for 2 d. D, 2μ-CDC28 suppresses CDC28-VF. All strains contain pGAL-MPS1. Wild-type (KH153) or CDC28-VF (KH181) 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.

Cdc28 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 hct1A 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 (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. 7B), showing that the CDC28-VF and clb2Δ mutations compromise Cdc20-dependent APC activity.

Earlier studies argued that Hct1 and Cdc20 were specificity factors for the APC, 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. 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 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 cells.

**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
Checkpoint-dependent Arrest

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 kinetochores 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.

Figure 8. Cdc20 binding to the APC is impaired in CDC28-VF. cdc15-2 (A DR1790) and cdc15-2 CDC28-VF (A DR1793) were grown overnight in YEP + 2% glucose at 23°C to log phase. Both strains contain an epitope-tagged Cdc20 (CDC20-myc12). The cultures were shifted to 37°C and after >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 immunoprecipitated with 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 APC were precipitated with the anti-Cdc26 antibodies as judged by Cdc23 levels (left) and equal amounts of cell lysate were used in the immunoprecipitation as judged by Cdc20-myc12 and Cdc28-HA levels (right, cell lysate).

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 cld2Δ and cks1-38. Do these four mutants, CDC28-VF, cdc28-1N, cld2Δ and cks1-38, have a common biochemical defect? cld2Δ 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., 1991; Kaiser 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 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 CDC28-F88G, a mutant in the ATP binding site of Cdc28, also has reduced specific activity (Bishop et al.,...
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 APC by modifying four of its subunits: Cdc16, Cdc23, Cdc27, and Apc1 (Bimé; Hershko et al., 1994; Peters et al., 1996; K otani et al., 1998, 1999; Patra and Unph, 1998). These proteins are phosphorylated during mitosis and the phosphorylated APC has greater Cdc20-dependent activity in vitro, whereas dephosphorylation of purified APC 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 APC is phosphorylated by Cdc28 in budding yeast, and that a defect in this phosphorylation causes reduced Cdc20-dependent APC activity and contributes to the CDC28-VF phenotype.

We would like to thank Doug Kellogg, Aaron Straight, Lena Hong, Sue Biggins, Sue Jaspersen, Rachel Tinker-Kulberg, Jeff Ubersax, Dave Morgan, Mike Mendenhall, Ray Dueshia, Bob Boorer, Andy Page, Phil Hieter, Wolfgang Zachariae, Kim Nasmyth, Jeremy Thorner, Peter Sorger, and Steve Reed for yeast strains, plasmids, and antibodies; Jeff Ubersax, Julia Charles, and David Morgan for sharing unpublished results; Jeff Ubersax, Sue Jaspersen, Dave Morgan, and the Murray Lab for critical reading of the manuscript; Jeremy M inshull, Bodo Stern, David Rudner, Alex Szidn, Julia Charles, Rachel Tinker-Kulberg, Hiroonri Funakibi, Sue Biggins, Marc Lenburg, and Dara Spatz Friedman for invaluable discussions and unwavering support.

This work was supported by grants from the National Institutes of Health and Human Frontiers in Science to A.W. Murray. A.D. Rudner was a pre-doctoral fellow of the Howard Hughes Medical Institute and K.G. Hardwick was a fellow of the Leukemia Society of America merica during this work.

Submitted: 28 March 2000
Accepted: 17 May 2000

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