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*Science* **279**, 1041 (1998);
DOI: 10.1126/science.279.5353.1041

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Budding Yeast Cdc20: A Target of the Spindle Checkpoint

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The spindle checkpoint regulates the cell division cycle by keeping cells with defective spindles from leaving mitosis. In the two-hybrid system, three proteins that are components of the checkpoint, Mad1, Mad2, and Mad3, were shown to interact with Cdc20, a protein required for exit from mitosis. Mad2 and Mad3 coprecipitated with Cdc20 at all stages of the cell cycle. The binding of Mad2 depended on Mad1 and that of Mad3 on Mad1 and Mad2. Overexpression of Cdc20 allowed cells with a depolymerized spindle or damaged DNA to leave mitosis but did not overcome the arrest caused by unreplicated DNA. Mutants in Cdc20 that were resistant to the spindle checkpoint no longer bound Mad proteins, suggesting that Cdc20 is the target of the spindle checkpoint.

The spindle checkpoint improves the fidelity of chromosome segregation by delaying anaphase until all chromosomes are correctly aligned on the mitotic spindle (1, 2). Mutants in the MAD (mitosis arrest deficient) and BUB (budding uninhibited by benzimidazole) genes inactivate the checkpoint (3, 4), and overexpressing components of the checkpoint can arrest cells with normal spindles in mitosis (5–7). The checkpoint prevents ubiquitination and destruction of at least two types of protein: the B-type cyclins, which activate the protein kinase activity of cyclin-dependent kinase (Cdk1, known as Cdc28 in budding yeast and Cdc2 in fission yeast), and a protein required to maintain the linkage of sister chromatids (Pds1 in budding yeast and Cut2 in fission yeast) (8–11). Ubiquitination is catalyzed by a multiprotein complex called the cyclosome or anaphase promoting complex (APC) (12–14). The reactions that activate the APC are not understood, but cyclin B and Pds1/Cut2 destruction depends on Cdc20 and Hct1/Cdh1, two evolutionarily conserved members of the WD (Trp-Asp) repeat family of proteins. Cdc20 preferentially promotes the destruction of Pds1/Cut2, and Hct1 promotes the destruction of B-type cyclins (15, 16). Unlike HCT1, CDC20 is an essential gene, and temperature-sensitive cdc20 mutants arrest in metaphase.

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References

2. Ets-1 fragments ΔN331 or ΔN280 (37) did not interact with GABPB 1 or 2 in electrophoretic mobility-shift assays when mixed or codified from B M urea.
3. In a model of Ets-1 bound to GABPs, the substitution of valine for Glu220 disrupts a hydrogen bond and the substitution leucine for Val115 results in a steric clash with neighboring hydrophobic residues.
4. Curve of the DNA was calculated for the 9 bp contacting GABPs with the program CURVES; R. Lavery and H. Sklenar, J. Biomol. Struct. Dyn. 6, 63 (1988).
16. We thank S. Soisson and E. Reisinger for continuous assistance; S. Prigge, D. Hammontree, and D. Leahy for programs; M. Blanchet and M. Amzel for x-ray room assistance; C. Ogata for beamline assistance; N. Pavletich for 33BP2 coordinates; and B. Graves for Ets-1 samples. This work was initiated during a sabbatical visit by S.L.M. in the laboratory of C.W. At that time S.L.M. was supported by the Carnegie Institute of Washington and the Howard Hughes Medical Institute. A.H.B., D.E.P., and C.W. were supported by the Howard Hughes Medical Institute. C.W. also received support from the David and Lucile Packard Foundation. Coordinates have been deposited at the Brookhaven Protein Data Bank (accession code 1awo).
17. 18 August 1997; accepted 18 December 1997.
A single wild-type sporodiplon, 3Mad1, 3Mad2, and 3Mad3 with Cdc20. Two-hybrid association. (A) Haploid strains containing fusions between the transcriptional activation domain and Cdc20 or Snf4 were crossed to strains containing fusions between the DNA-binding domain and Mad proteins or Snf1, and the resulting diploids were assayed for β-galactosidase activity. Values are shown in Miller units and are the average of three independent crosses. The fusions were constructed in the vectors pAS1-CYH2 (DNA binding domain) and pACTII (transcriptional activation domain) (32), and all contained the full coding region, except for the Mad1 fusion, which contains amino acids 313 to 750 of Mad1. The SNF1 and SNF4 fusions are control fusions to proteins involved in regulating sucrose metabolism. (B) Cell cycle regulation of Mad-Cdc20 interactions. Coimmunoprecipitation of Cdc20 with Mad2 and Mad3. Strains containing a hemagglutinin (HA) epitope–tagged version of Cdc20 were lysed, then immunoprecipitated with antibodies to the HA epitope, and the immunoprecipitates were analyzed by protein immunoblotting with antibodies to Mad2, Mad3, or HA. Extracts were from exponentially growing cells, arrested in G1 by treatment with α-factor, arrested in mitosis by treatment with nocodazole, or cells arrested in mitosis by the temperature-sensitive cdc26Δ mutant (18, 19). The polyclonal antibodies to Mad3 recognize a background protein directly below the Mad3 protein. (C) Effect of mad mutants on Mad-Cdc20 interactions. Exponentially growing mad1Δ, mad2Δ, and mad3Δ strains containing HA-tagged Cdc20 were lysed, and the lysates were analyzed for the interactions of Mad2 and Mad3 with Cdc20.

Fig. 2. Cdc20 overexpression overcomes the spindle and DNA damage checkpoints. The indicated strains were subjected to (A) spindle depolymerization by nocodazole, (B) DNA damage caused by shifting the cdc13-1 mutant to 33°C, or (C) inhibition of DNA replication by hydroxyurea. For measurement of rebudding and DNA content (morphological and biochemical criteria for exit from mitosis), cells were grown in raffinose-containing medium, arrested by drug or temperature treatment for 165 min, and then treated with 2% galactose to induce expression of Cdc20 from the GAL1 promoter. Samples were taken at the indicated times after galactose addition. Arrows indicate the 1N DNA content of G1 cells and 2N content of G2 cells. The slow rightward shift of the DNA peak in nocodazole-treated cdc23-1 cells during the experiment is due to mitochondrial DNA replication. For measurements of cell viability, galactose was added to cells growing exponentially in raffinose-containing medium at the same time that nocodazole or hydroxyurea was added or the cells were shifted to 33°C. At the indicated times, cells were removed and tested for their ability to give rise to colonies on glucose-containing medium.
Cdc20 suggests that Cdc20 is the target for the spindle checkpoint. We therefore tested whether overexpressing Cdc20 overcame any cell cycle checkpoints (23). Cells overexpressing Cdc20 from the GAL1 promoter were defective in two checkpoints: They no longer arrested in mitosis in response to spindle depolymerization (Fig. 2A) or to the DNA damage caused by the cdc13-1 mutation [Fig. 2B and (24, 25)]. Overexpression of Cdc20 bypasses checkpoints by activating the APC, because combining the cdc23-1 mutation, which disrupts APC activity, with GAL-CDC20 restores the mitotic arrest to cells with damaged spindles or DNA. Overexpressing Cdc20 does not overcome the cell cycle arrest caused by hydroxyurea, an inhibitor of DNA synthesis (Fig. 2C). These results are consistent with the idea that the spindle and DNA damage checkpoints arrest cells in mitosis by inhibiting Cdc20 and suggest that the DNA replication checkpoint uses another mechanism to arrest the cell cycle.

If Cdc20 is the target of the spindle checkpoint, it should be possible to isolate dominant mutations in Cdc20 that no longer respond to the checkpoint. Overexpression of MPS1, a component of the checkpoint, arrests cells in mitosis even though their spindle is still fully functional (5). We mutagenized the CDC20 gene, selected for mutants that overcame the mitotic arrest caused by Mps1 overexpression, and analyzed four of these mutants in detail (26). Mutant forms of Cdc20, like mad and bub mutants (5), allowed cells overexpressing Mps1 to proliferate more rapidly than control cells (Fig. 3A). The Cdc20 mutants also increased the rate at which cells exited mitosis and died after treatment with nocodazole (Fig. 3B). The checkpoint-resistant Cdc20 mutants also weaken the DNA damage checkpoint, although this effect is somewhat variable (21). The checkpoint-resistant mutants complement the temperature-sensitive growth defect of cdc20-1 strains. In the cdc20-1 strains that carry the mutant plasmids, the spindle checkpoint is inactive at 37°C, demonstrating that the checkpoint defect is not due to a dominant negative activity of the Cdc20 mutants (21). The checkpoint-resistant Cdc20 mutants greatly diminished the binding of Mad2 and Mad3 to Cdc20 (Fig. 3C) but had no effect on the overall level of Mad1, Mad2, or Mad3 in the cell (21). For all four mutants, the checkpoint-resistant phenotype is conferred by the mutations in the region of Cdc20 shown by Kim et al. to be required for interaction with Mad2 in fission yeast (7). Sequencing this region revealed that all the mutants had changes in a short region of Cdc20 that correspond to the region that contains the checkpoint-resistant mutation in Slp1 (Fig. 3D). Hoyt and Schott have also isolated a dominant allele of CDC20 that bypasses the spindle assembly checkpoint but not the DNA damage checkpoint (25). It is not known if this mutant affects the interaction between Cdc20 and the Mad proteins.

The idea that the spindle checkpoint specifically inhibits Cdc20 is strengthened by comparing the sequences of Cdc20 and Hct1. These proteins have homologs in fission yeast, plants, and animals, and probably conserve the functional difference between Cdc20 and Hct1; Cdc20 is required for the proteolysis that drives the metaphase-to-anaphase transition, and Hct1 is required for cyclin B proteolysis during G2 (15, 16). Members of the Cdc20 family have conserved the region defined in fission yeast as the Mad2 interaction region (7), but members of the Hct1 family lack this homology. It has been suggested that Mad2 inhibits exit from mitosis by binding directly to the APC (6), but we have been unable to detect binding of Mad2 to the APC in yeast (21).

We do not yet understand how the checkpoint regulates Cdc20 activity. Although Mad2 and Mad3 are bound to Cdc20, this association does not change on activation of the checkpoint. Perhaps

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**Fig. 3.** Dominant Cdc20 mutants that overcome the spindle checkpoint. (A) Cdc20 mutants proliferate despite overexpression of Mps1. Strain LH317 (MATa ade2-1 his3-11,15 leu2-3,112 ura3 TRP1 GAL-1) containing centromeric plasmids with wild-type or mutant CDC20 were grown to saturation and diluted onto galactose-containing plates. After 16 hours, the number of cell bodies plus buds in 100 microcolonies was counted. (B) Cdc20 mutants re-bud and die rapidly when treated with nocodazole. Exponentially growing cells of a MATa bar1 Δ derivative of W303 containing centromeric plasmids with wild-type or mutant CDC20 were treated with nocodazole (15 μg/ml) for 0, 2, 3, 25, and 6 hours and then plated for viability. Values are expressed as a percentage of the viability before nocodazole addition. (C) Checkpoint-resistant Cdc20 mutants have diminished binding of Mad2 and Mad3. Exponentially growing cultures containing HA-tagged wild-type or mutant CDC20 were lysed, lysates were immunoprecipitated with antibodies to the HA epitope, and the immunoprecipitates were analyzed by protein immunoblotting with antibodies to Mad2, Mad3, or HA. (D) Sequence changes in dominant, checkpoint-resistant Cdc20 alleles. Shown are changes from the wild-type sequence in four mutants and the comparison with the sequence of Slp1 (the fission yeast homolog of Cdc20) and the checkpoint-resistant Slp1 mutant described by Kim et al. (7). The CDC20-120 allele includes two changes outside this region, Trp206→Ser and Trp247→Ile; we do not know if these changes are sufficient to confer the mutant phenotype. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; and Y, Tyr.
modification of one of the Mad proteins converts it into a form that can inhibit as well as bind to Cdc20. Although we have not detected modification of Mad2 and Mad3, Mad1 becomes hyperphosphorylated on activation of the checkpoint (8). An alternative possibility is that other, undetected proteins show checkpoint-dependent binding to Mad2 or Mad3 and inhibit Cdc20 activity.

Overexpression of Cdc20 and dominant Cdc20 mutants interferes with both the DNA damage and spindle checkpoints. The role of Cdc20 in the DNA damage checkpoint is independent of the Mad proteins because mad mutants have an intact DNA damage checkpoint (27). We speculate that in all eukaryotes the spindle checkpoint prevents the onset of anaphase by inhibiting Cdc20. In contrast, inhibition of mitotic exit by the DNA damage checkpoint is likely to be confined to organisms like budding yeast that lack a clearly defined transition between G2 and mitosis. In animals and fission yeast, DNA damage can arrest cells in G2, thus keeping them from entering mitosis and condensing their chromosomes. In budding yeast, the mitotic spindle assemble normally in cells with damaged or unreplicated DNA. Thus, the checkpoints that detect these defects must be able to prevent the exit from rather than the entry into metaphase. The budding yeast Pds1 protein may be involved in this evolutionary shift in the target of the DNA damage checkpoint. Destruction of Pds1 and its fusion yeast homolog (Cut2) are required to separate sister chromatids (9, 10) and in budding yeast Pds1 is required for the DNA damage checkpoint in G2 (28). One explanation for this dual requirement is that Pds1 acts both as a substrate and an inhibitor of the APC, with inhibitory function being independent of the destruction box. In undamaged cells, Pds1 binding to the activated APC would partially inhibit APC activity, but because the APC can target Pds1 for destruction, cells could rapidly escape this inhibition. DNA damage would induce modifications of Pds1 that increased its ability to inhibit the APC; consequently, Pds1 and mitotic cyclins would be stable, sister chromatids would not separate, and cells would remain in metaphase. This model explains why an indestructible form of Pds1 arrests budding yeast in metaphase. A similar mutation in Cut2, the fission yeast homolog of Pds1, prevents sister chromatid separation but not cyclin B destruction and the exit from mitosis. This difference correlates with the different organization of the cell cycle in the two yeasts.

REFERENCES AND NOTES

4. R. Li and A. W. Murray, ibid., p. 519.
17. All experiments in this study were done in strains isogenic to W303 with standard media (29). Wild-type or cdc26 strains contain the centromeric plasmid pLH68, which contains the CDC20 gene with a triple hemagglutinin (HA) tag and a six-histidine tag at its COOH-terminus and the URA3 gene. Cultures were grown in medium without uracil until mid-log phase, transferred to rich medium without further treatment or with the following treatments relative to 1 µg/mL to induce G2 arrest or with nocodazole (15 µg/mL) to activate the spindle checkpoint; in cdc26 strains, the cells were shifted to 37°C to inactivate the APC. Cells were harvested and lysed, and lysates were immunoprecipitated and protein immunoblotted as described (19), with the exception that the lysing buffer contained 50 mM β-mercaptoethanol instead of 1 mM sodium vanadate.
20. A. Amon, unpublished data.
23. The plasmid pCM4 derived from the plasmid pMAP128 (a/a bar1/bar1 mec1-1/mec1-1 CDC28-VF::LEU2/CDC28-VF::LEU2) was transformed into strains L317 (MATa ade2-1 his3-11,15 leu2-3,112 ura3 TRP1 GAL-MPS1) and a MATa bar1 derivative of W303. For microcolony assays and rebudding, cultures were grown to saturation in URA-glucose medium, diluted 1:1,000, and sonicated to break up cell clumps, and 5 µl was spotted on YEP 2% galactose plates (for transformants in L317) or YEP 2% glucose plus benonyl plates (10 µg/mL) for transformants in MATa bar1). At the indicated times, the number of cells plus buds was counted in at least 100 microcolonies. To monitor nocodazole induced death, we spotted cultures grown to saturation on URA-glucose medium into YEP-glucose medium, then incubated them at 30°C for 16 hours before adding nocodazole to 15 µg/mL. Cells were plated for viability on URA-glucose medium, then were grown on YEP-glucose medium to induce nocodazole addition, and 2, 3, 24, and 6 hours later.
28. A. Rudner, D. A. A.Straight for yeast materials, and members of our labs for valuable discussions. We are grateful to T. Matsumoto for encouragement, helpful discussions, and for completed results. Supported by a National Science Foundation Fellowship (L.H.H.), a grant from NIH (A.A.), and grants from NIH, the March of Dimes, and the David and Lucile Packard Foundation (A.W.M.).
29. 11 December 1997; accepted 14 January 1998.

1044 SCIENCE • VOL. 279 • 13 FEBRUARY 1998 • www.sciencemag.org