Cell Cycle Arrest in cdc20 Mutants of Saccharomyces cerevisiae Is Independent of Ndc10p and Kinetochore Function but Requires a Subset of Spindle Checkpoint Genes

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ABSTRACT

The spindle checkpoint ensures accurate chromosome segregation by inhibiting anaphase onset in response to altered microtubule function and impaired kinetochore function. In this study, we report that the ability of the anti-microtubule drug nocodazole to inhibit cell cycle progression in Saccharomyces cerevisiae depends on the function of the kinetochore protein encoded by NDC10. We examined the role of the spindle checkpoint in the arrest in cdc20 mutants that arrest prior to anaphase with an aberrant spindle. The arrest in cdc20 defective cells is dependent on the BUB2 checkpoint and independent of the Cdc20, BUB3, and MAD spindle checkpoint genes. We show that the lesion recognized by Bub2p is not excess microtubules, and the cdc20 arrest is independent of kinetochore function. We show that Cdc20p is not required for cyclin proteolysis at two points in the cell cycle, suggesting that CDC20 is distinct from genes encoding integral proteins of the anaphase-promoting complex.

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The accurate transmission of genetic information is governed by feedback mechanisms to ensure the strict order of events during the cell cycle. Checkpoint controls prevent late events in the cell cycle from being initiated until the completion of earlier events. Checkpoint genes are defined empirically by mutations that relieve dependency relationships, thereby uncoupling mitotic processes (Hartwell and Weinert 1989). The DNA-responsive checkpoints ensure that chromosome segregation is not initiated in the presence of damaged DNA or incompletely replicated DNA. Cells are capable of responding to multiple types of lesions and arrest at distinct points during the cell cycle, depending on when the damage is encountered (G1/S, intra-S, and G2/M, mid-anaphase) (Lyall and Weinert 1996; Yang et al. 1997). A distinct checkpoint monitors the mitotic spindle. The spindle checkpoint assures that the metaphase to anaphase transition is not initiated until the proper assembly of the mitotic spindle and the bipolar attachment of each chromosome to the spindle are established (Wells 1996). In Saccharomyces cerevisiae, the spindle checkpoint function has been defined by mutations that permit cells to proceed in mitosis in the presence of the microtubule depolymerizing drugs benomyl and nocodazole. The original genetic screens identified three MAD (mitotic-arrest-deficient) and three BUB (budding-uninhibited-by-benzimidazole) genes that are required to arrest cell division when spindle structure is disrupted (Hoyt et al. 1991; Li and Murray 1991). The identification of homologues of BUB2 and MAD2 in S. pombe (cdc161, mad21), MAD2 in Xenopus and human cells, and BUB1 in murine cells suggests that spindle checkpoint functions have been conserved throughout evolution (Chen et al. 1996; Fankhauser et al. 1993; He et al. 1997; Li and Benezra 1996; Taylor and McKeon 1997). Recent work from several labs suggests that the lesional monitored by the MAD and BUB spindle checkpoint genes is impaired kinetochore function (Pangilinan and Spencer 1996; Wang and Burke 1995; Wells 1996). It has recently been proposed that assembly of the budding yeast kinetochore complex may be necessary for the spindle checkpoint to function (Sorger et al. 1995; Wells 1996).

Temperature-sensitive cdc20 mutants are defective in the microtubule-dependent processes of karyogamy, nuclear transit, and chromosome segregation (Palmer et al. 1989; Sethi et al. 1991). Anti-α-tubulin immunofluorescence indicates that the cdc20 arrest, before chromosome segregation, is coincident with a short, intensely stained spindle, suggesting a role for Cdc20p in modulating microtubule function (Sethi et al. 1991). The increased fluorescence correlates with an increase in the number of intranuclear microtubules as revealed by electron microscope serial reconstruction of cdc20 spindles (O'Toole et al. 1997). Reciprocal shift experiments indicate that the CDC20-dependent step and the nocodazole-sensitive steps are interdependent (Sethi 1993). One simple interpretation is that the abnormal spindle structure in cdc20 defective cells activates BUB- and MAD-dependent spindle checkpoints causing cells to arrest prior to anaphase.

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The separation of chromatids and the exit from mitosis requires ubiquitin-mediated proteolysis (Holloway et al. 1993; Surana et al. 1993). Recent work from Xenopus, human cells, Spisula, and budding yeast identified a multisubunit complex, the anaphase promoting complex (APC), which is required for anaphase progression and the proteolysis of mitotic regulatory proteins (Irniger et al. 1995; King et al. 1995; Peters et al. 1996; Sudakin et al. 1995; Tugendreich et al. 1995; Zachariae et al. 1996). Mutations in genes encoding components of the APC result in arrest with an uninduced nucleus at a stage indistinguishable from thecdc20 arrest (Iringer et al. 1995; Lamb et al. 1995; Setti et al. 1991). Recently, the Drosophila melanogaster homolog of CDC20, fizzy, has been implicated in cyclin degradation during mitosis (Dawson et al. 1994). A different interpretation of the arrest incdc20 mutants is that it is not a consequence of the MAD - and BUB-dependent feedback controls, but rather may be a consequence of defective mitotic cyclin proteolysis.

In this study, we show that arrest incdc20 mutants is independent of kinetochore function. We show that arrest is under control of the EB2 gene but independent of the other MAD and BUB checkpoint genes. We report that B-cyclin protein, Clb2p, does not require Cdc20p for degradation at two points in the cell cycle, suggesting that Cdc20p is not required for mitotic cyclin proteolysis. We discuss the possible role ofcdc20 in regulating the metaphase to anaphase transition in the context of these and other recent results.

MATERIALS AND METHODS

Strains and media: The yeast strains and plasmids used in this study are listed in Tables 1 and 2, respectively. Strains were constructed by standard genetic techniques using rich (YM-1 and YEPD) and synthetic media (SC) (Hartwell 1967; Sherman et al. 1986). Unbudded cells were isolated by growth to stationary phase for 2 days in rich (YM-1) or synthetic medium lacking uracil (SC-Ura) supplemented with 2% raffinose. Nocodazole (Sigma Chemical Co., St. Louis, MO) was used at a concentration of 15 μg nocodazole per ml in a final concentration of 1% dimethyl sulfoxide. Yeast transformations were performed by the "PLATE" (polyethylene glycol 4000, lithium acetate, Tris, EDTA) method described by Elble (1992). The DNA content of cells was determined by flow cytometry of propidium iodide (Calbiochem-Novabiochem Corp., La Jolla, CA) stained cells using a Becton Dickinson cell sorter as described previously (Smith 1991).

Strain construction: Strain 1906 (cdc20-1 mad1::HIS3) was constructed by transforming strain 1739-9-2 (cdc20 his3) with EcoRV and SacI digested plasmid pKH149 (Hardwick and Murray 1995). Strain 1370-3-1-3 (cdc20-1 mad2::URA3) was constructed by transforming the diploid strain 1370 with HindIII and XhoI digested plasmid pRC10.1 (R.-H. Chen and A. W. Murray, personal communication) and identifying Ura" spores from dissected tetrads. Strain 1370-3-1-4 (cdc20) is a segregant from the same tetrad as 1370-3-1-3 (cdc20 mad2). Disruptions of MAD1 and MAD2 were confirmed by PCR using primers flanking the auxotrophic markers of the individual disruptions.

Strains 1907 and 1908 are rho" derivatives of strains H20C1B1 and MAY2099, respectively. rho" derivatives were constructed by growth in ethidium bromide as described (Sherman et al. 1986). Elimination of mitochondrial DNA was confirmed by inability to grow on medium containing glycerol and by DNA staining with DAPI (4',6-diamidino-2-phenylindole). Strain 1749-33A (ndc10-1 GFP-lacZ::His3 lacO::LEU2) and strain 1798 (NDC10 GFP-lacZ::His3 lacO::LEU2) were constructed by transforming strain 1749-33 (ndc10-1 lacZ::his3) and W303 (NDC10 lacZ::his3) with pAFP559 linearized with EcoRV. Leucine prototrophs were chosen and subsequently transformed with pAF5144 cut with Heli. Uracl prototrophs were chosen and designated as 1749-33A and 1798. All other strains were derived by standard crosses.

GFP sister chromatid separation assay: Cells that were grown to mid-logarithmic phase in YM-1 were resuspended in SC-HIS medium supplemented with 10 mm 3-aminotriazole for 30 min to induce the H3S promoter. The cells were washed by centrifugation and resuspended in YM-1 containing 15 μg of nocodazole per ml nocodazole and incubated at the restrictive temperature of 37°C. Samples were removed every 30 min and fixed in 3% formaldehyde for 15 min at room temperature. The cells were washed in 65 mm NaPO4, 5 mm MgCl2, mounted on slides, and GFP staining was visualized using standard FITC filters.

Indirect immunofluorescence and photomicroscopy: Anti-tubulin immunofluorescence and DNA staining with DAPI were performed essentially as described previously with the exception of a shorter fixation time (Adams and Pringle 1984). Cells were fixed in 3.7% formaldehyde for 15 min at 37°C. The monoclonal rat antitubulin antibody YOL 1/34 and secondary goat-anti-rabbit antibody (FITC-conjugated; Serotec, Washington, DC) were used at a dilution of 1:50 in phosphate buffered saline (PBS) containing 1% bovine serum albumin and 0.1% sodium azide. Cells were cells arrested in nocodazole and incubated for the arrest in nocodazole. We diluted the cells into SC-Ura medium containing raffinose for 2 days. We diluted the cells into SC-Ura medium containing raffinose and the proteolysis of mitotic regulatory proteins forming strain 1749-33 (ndc10-1 lacZ::his3) and W303 (NDC10 lacZ::his3) with pAFP559 linearized with EcoRV. Leucine prototrophs were chosen and subsequently transformed with pAF5144 cut with Heli. Uracl prototrophs were chosen and designated as 1749-33A and 1798. All other strains were derived by standard crosses.

Histone H1 kinase assay: Histone H1 kinase activity was measured in crude protein extracts as the ability to phosphorylate histone H1 (Boehringer Mannheim Corp., Indianapolis, IN) as described by Surana et al. (1991). Bovine serum albumin (BSA) was used as a standard for protein concentration measurements using the Bradford method (Bio Rad Labs., Hercules, CA). Equal amounts of total cell protein were used in the kinase reactions and equal gel loading was confirmed by Coomassie staining prior to phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA).

Clb2p stability: We enriched for unbudded cells by growth to stationary phase in SC-Ura containing raffinose for 2 days. We diluted the cells into SC-Ura medium containing raffinose plus α factor and incubated the cells until 90% of the cells displayed the morphology of pheromone-arrested cells. Bar1 cells were arrested in α factor (Sigma Chemical Co.) at a concentration of 0.5 μg/ml from a stock solution of 1 mg α factor per ml in PBS. The GAL10 promoter was induced by adding galactose (2%) to cells pregrown in 2% raffinose. Galactose was added to the medium, and cells were incubated at 36°C, the restrictive temperature forcdc20 mutants, for 2 hr to induce Clb2p synthesis. To examine Clb2p incdc20 cells at G2/M, cells from strain 1904 were grown to midlogarithmic phase in SC-Ura supplemented with 2% raffinose. The culture was shifted in half, and galactose (2%) was added to one half of the culture, and additional raffinose (2%) added to the uninduced culture. The cells were incubated for 2 hr at the restrictive temperature.

Cell extracts and immunoblotting: Crude cell extracts were prepared as described by Surana et al. (1993). Proteins from...
## TABLE 1

### Strains used in this study

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<th>Strain</th>
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</tr>
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50 µg of cell extract were separated on 10% SDS-PAGE gels. Proteins were transferred to nitrocellulose membrane Hybond enhanced chemiluminescence (ECL; Amersham Life Science, Arlington Heights, IL) in transfer buffer specified by Trans-Blot SD system (Bio Rad Labs.). Blots were blocked for 1 hr at room temperature in 10% dry nonfat milk in PBS containing 0.1% Tween-20 (PBS-T). The blots were washed in PBS-T at room temperature according to the ECL Western blot protocol (Amersham Life Science). To detect Ctb2-HAp, the mouse monoclonal anti-HA peroxidase conjugated antibody, 12CA5 (Boehringer Mannheim Corp.), was used at a dilution of 1:1000 in PBS-T containing 10% milk and incubated overnight at 4°C. To reprobe the membrane, bound antibodies were removed by extensive washing in 2% SDS in water followed by several rinses with water and PBS-T. To detect Tub2p, the rabbit monoclonal primary antibody FY124 (Bond et al. 1986).

## TABLE 2

### Plasmids used in this study

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<td>mad2::URA3</td>
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<td>GAL10::CLB2 URA3 3XHA</td>
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<td>pAF5144</td>
<td>A. F. Straight and A. W. Murray</td>
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</table>
was diluted 1000-fold in PBS-T with 10% milk and incubated overnight at 4°C. The blot was washed with PBS-T and incubated with anti-rabbit IgG-horseradish peroxidase conjugated secondary antibody (Amersham Life Science) diluted 15,000-fold in PBS-T containing 10% milk for 1 hr at room temperature. The blots were washed after incubation with peroxidase-conjugated antibodies and immunocomplexes detected by ECL according to the manufacturer’s instructions (Amersham Life Science).

RESULTS

NDC10 is required for spindle checkpoint function:
Four subunits define the essential yeast centromere binding factor, CBF3 (Lechner and Ortiz 1996; Pluta et al. 1995). Mutations in the structural genes encoding the p58 (CTF13) (Doheny et al. 1993), the p64 (CEP3/ CBF3B) (Lechner 1994; Strunnikov et al. 1995), and the p23 (SKP1) (Bai et al. 1996; Connelly and Hieter 1996) components of CBF3 arrest cells prior to anaphase. The arrest in response to altered Ctf13p function is under the control of the spindle checkpoint, suggesting that the yeast spindle checkpoint monitors some aspect of kinetochore activity (Pangilinan and Spencer 1996; Wang and Burke 1995). Surprisingly, mutations in the 110-kD component of CBF3, encoded by NDC10 (NDC10/CBF2/CTF14/CEP2), do not cause cells to arrest (Doheny et al. 1993; Goh and Kitt martin 1993; Jiang et al. 1993; Strunnikov et al. 1995). Cells completely mitotic, resulting in an asymmetric segregation of DNA. Therefore, the spindle checkpoint is unable to restrain mitosis in cells where kinetochore function has been compromised by a mutation in NDC10. Sorger et al. (1995) propose that proper assembly of CBF3-DNA complex is necessary for spindle checkpoint function. Therefore, activating the spindle checkpoint with nocodazole should not inhibit cell cycle progression in ndc10 cells.

We determined the ability of a microtubule inhibitor to activate the spindle checkpoint in the absence of NDC10 function by analyzing DNA content in ndc10 cells treated with nocodazole. Cells from strain JK418 (ndc10-1) and strain W303 (WT) were grown to midlogarithmic phase and shifted to the restrictive temperature in the presence of nocodazole. We confirmed that the nocodazole treatment was effective in eliminating microtubules by analyzing samples of cells at each time point by antitubulin immunofluorescence (data not shown). In each case, greater than 90% of the cells showed tubulin staining only as punctate foci at the spindle pole bodies, indicative of completely effective nocodazole treatment (Jacobs et al. 1988). Wild-type cells arrested in the cell cycle with a 2N content of DNA. Treatment with nocodazole was unable to inhibit cell cycle progression in ndc10 cells (Figure 1). ndc10 cells, in the absence of nocodazole, showed a population of cells with a DNA content of greater than 2N as described previously (Figure 1) (Goh and Kitt mart in 1993). Similarly, in ndc10 cells treated with nocodazole, the DNA profile displayed a peak of greater than 2N (Figure 1). Therefore, ndc10-1 is epistatic to nocodazole, and activating the spindle checkpoint with nocodazole does not inhibit cell cycle progression in ndc10 cells.

To confirm that nocodazole was unable to prevent anaphase onset in ndc10 cells, we examined the cohesion of sister chromatids in nocodazole-treated ndc10 cells. Sister chromatid separation does not require microtubules, and cells defective for the spindle checkpoint separate sister chromatids in the presence of nocodazole (Straight et al. 1996). We used a simple cytological assay based on the use of green fluorescent protein (GFP) as described by Straight et al. (1996) to visualize yeast chromosome separation. Cells treated with nocodazole have sister chromatids that are in such close proximity that they appear as a single spot of GFP staining. However, when sister chromatids separate in the absence of microtubules, there are two foci of GFP staining (Straight et al. 1996). We treated cells from strains 1798 (NDC10 GFP-lacI::HIS3 lacO::LEU2) and 1749-33A (ndc10-1 GFP-lacI::HIS3 lacO::LEU2) with nocodazole and grew cells at the restrictive temperature for ndc10-1. We determined the extent of sister chromatid separation as the percentage of cells containing two GFP-staining foci. The percentage of cells containing separated sister chromatids was low and did not increase in NDC10 cells. However, the percentage of cells containing two GFP-staining foci increased over time when Ndc10p was inactivated at the restrictive temperature in cells from strain 1749-33A (Figure 2). These data suggest that Ndc10p function is required for the spindle checkpoint to delay anaphase onset.

cdc20 is epistatic to ndc10: We used the ndc10-1 muta-
We analyzed spindle morphology in checkpoint double mutants. Spindle structure and cell morphology are unaffected at high temperatures in checkpoint mutants (Pangilinan et al. 1996; data not shown). Therefore, we grew cells from strains MAY1787 (cdc20 bub1), 1908 (cdc20 bub2), MAY2113 (cdc20 bub3), 1906 (cdc20 mad1), 1370-3-1-3 (cdc20 mad2), and H20C1B1 (cdc20) to midlogarithmic phase and incubated cells at the restrictive temperature for 4 hr. We analyzed the cell cycle arrest by anti-tubulin immu-

**Table 3**

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<th>Treatment (4 hr)</th>
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<th>S&lt;sup&gt;B&lt;/sup&gt;</th>
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<td>26</td>
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<sup>1</sup>U, unbudded.
<sup>2</sup>S<sub>B</sub>, small budded.
<sup>3</sup>L<sub>B</sub>, large budded.
<sup>4</sup>M<sub>B</sub>, multibudded.

Figure 2.—Sister chromatid cohesion in ndc10 cells. Cells from strains 1798 (NDC10 GFP-lacI::HIS3 lacO::LEU2) (■) and 1746-33A (ndc10-1 GFP-lacI::HIS3 lacO::LEU2) (○) were treated with 15 μg nocodazole per ml at the restrictive temperature. Two GFP-staining foci in the unseparated nuclei of large budded cells were scored as having undergone sister chromatid separation. Time points were taken every 30 min for 4 hr.

Figure 3.—Effect of cdc20 on cells deprived of NDC10 function. Histone H1 kinase activity in cells from strain H20C1B1 (cdc20-1), 492 (ndc10-169), 796-8-2 (cdc20-1 ndc10-169), JK418 (ndc10-1), and 1738-4-2 (cdc20-1 ndc10-1) after a 4-hr incubation at the restrictive temperature.
no fluorescence and DNA staining with DAPI. In each double mutant combination except cdc20 bub2, greater than 70% of the cells contained a short spindle with an undivided nucleus characteristic of the preanaphase arrest observed in cdc20-defective cells (Setti et al. 1991). These results agree with Hoyt et al. (1991) that cdc20-defective cells do not require BUB1 and BUB3 to arrest cell division. However, in cdc20 bub2 mutants, only 46% of cells arrested cell division with short spindles concomitant with an accumulation of unbudded cells (25%) and cells with unusual microtubule-containing structures (29%) (Figure 4). The increase in unbudded cells suggests that some cdc20 cells complete nuclear division and cytokinesis in the absence of BUB2 function. Many of the unbudded cells contained microtubule structures of unusual length or morphology for an unbudded cell. We observed a population of cells with multipolar spindles, indicating that the spindle pole body cycle was uncoupled from nuclear division.

Cells defective in the spindle checkpoint initiate new rounds of budding in the presence of microtubule depolymerizing drugs (Hoyt et al. 1991; Weiss and Winey 1996; Straight et al. 1996). We examined the budding morphology of cdc20 and checkpoint double mutants to determine if the spindle checkpoint genes were required to prevent execution of a new budding cycle in cdc20-arrested cells. We grew cells from strains MAY1787 (cdc20 bub1), 1908 (cdc20 bub2), MAY2113 (cdc20 bub3), 1906 (cdc20 mad1), 1370-3-1-3 (cdc20 mad2), and 1370-3-1-4 (cdc20) displaying multiple buds (≥3) after shifting to the restrictive temperature. (B) Budding morphology in cells from strains 1908 (cdc20 bub2) and 405-1 (cdc20) grown for 4 hr at the restrictive temperature. Bar, 5 μm.

We did not observe the continued budding in cdc20 bub2 mutants until several hours after shift to the restrictive temperature. Therefore, we examined the kinetics of cell cycle arrest in cells from strain 1908 (cdc20 bub2) and strain 405-1 (cdc20). We analyzed spindle morphology over time in cells released from α-factor synchronization and grown at the restrictive temperature. Samples were harvested each hour and fixed for antitubulin immunofluorescence. We determined the percentage of cells containing short spindles at medial nuclear division (Figure 6A). By 2 hr, 75% of the cells from both strains had short spindles. Wild-type cells normally complete
Cell Cycle Arrest in cdc20 Mutants

Figure 6.—cdc20 bub2::URA3 mutants cannot maintain a mitotic arrest. (A) Percentage of cells displaying medial nuclear division (MND) spindles in cells incubated at 36°C. Cells from strain 1908 (cdc20 bub2) and strain H20C1B1 (cdc20) were released from α-factor arrest and grown at the restrictive temperature. Cells were removed and fixed in formaldehyde for antitubulin immunostaining each hour over a 6-hr time period. (B) Viability in cells from strains 1907 (cdc20) and 1908 (cdc20 bub2) incubated at the restrictive temperature. In (A) and (B): □, cdc20 bub2::URA3 and ○, cdc20.

Two cell divisions at this temperature (36°C), suggesting that cdc20 bub2 mutants are competent to arrest for some time. However, over extended periods of time, the percentage of short spindles declined in strain 1908 (cdc20 bub2). In contrast, cells from strain 405-1 (cdc20) displayed a high percentage of short spindles throughout the extended incubations. These results suggest that cdc20 bub2 mutants were competent to establish a mitotic arrest and delay anaphase transiently but were unable to maintain the mitotic arrest.

We measured the viability over extended incubations to determine if a loss of viability correlates with the cell cycle delay. Cells from strain 1908 (cdc20 bub2) and strain 1907 (cdc20) were grown to midlogarithmic phase, diluted, and spread onto YEPD plates. The plates were incubated at 36°C, and at 1 hr intervals plates were moved to the permissive temperature to determine the number of viable colonies. Figure 6B shows that cdc20 bub2 cells maintain high viability for 2 hr and then decrease in viability as compared to cdc20 cells. These data suggest that the inviability is associated with failure to arrest in the cell cycle. We conclude that the ability to maintain a cell cycle arrest in cdc20 cells is dependent on BUB2 and independent of other spindle checkpoint genes.

BUB2 does not arrest cells in response to microtubule overassembly: The unique requirement for BUB2 in executing a cdc20 arrest prompted us to investigate whether the excess numbers of microtubules in the spindle of cdc20 cells constitutes a distinct perturbation that induces a BUB2-dependent arrest in the cell cycle. There are cold-sensitive lethal mutations in the TUB1 α-tubulin gene that cause cells to arrest before anaphase with excess microtubules (Schatz et al. 1988). We used these cold-sensitive alleles of TUB1 to construct tub1 bub2 double mutants and asked whether BUB2 was required to arrest cells with excess microtubules induced by the mutations in TUB1. We tested bub2 in combination with three different TUB1 alleles (tub1-730, tub1-758, and tub1-741) that cause varying degrees of excess microtubules (Schatz et al. 1988). We measured viability in tub1 bub2 mutants after growth for extended times at the restrictive temperature (24 and 48 hr growth at 11°C). Wild-type cells normally complete 4 cell cycles in 48 hr at this temperature (Schatz et al. 1988). We did not detect a difference in viability between tub1 single mutants and tub1 bub2 double mutants, suggesting that the lesion recognized by BUB2 is not simply excess microtubules.

APC function is not eliminated in cdc20 mutants: Our data show that the BUB2-dependent arrest in cdc20 mutants is independent of most spindle assembly checkpoint genes and that BUB2 does not respond to overassembled microtubules. This suggests that the BUB2-dependent arrest in cdc20 is due to some other function that is lacking in the mutant. A role for CDC20 in ubiquitin-mediated proteolysis of cyclins has been proposed based primarily on the implication of the Drosophila homologue of CDC20, fizzy, in cyclin proteolysis (Dawson et al. 1995; Yamamoto et al. 1996). To determine whether the arrest in response to loss of CDC20 function is due to impaired cyclin proteolysis, we measured Clb2p...
stability in cells defective for Cdc20p function. Ubiquitin-mediated destruction of Clb2p is activated at the metaphase to anaphase transition and persists until G1 when cyclins accumulate (Amon et al. 1994). Clb2p is unstable in a-factor arrested cells because of APC activity (Amon et al. 1994; Irniger et al. 1995). We examined Clb2p stability in cdc20-defective cells in strain 1904 (bar1 cdc20 pGAL10::CLB2-3XHA) previously arrested by treatment with a factor. We also measured Clb2p stability in cells arrested before anaphase at the CDC20-dependent step. Clb2p was measured in total cell protein extracts by immunoblotting with an anti-HA monoclonal antibody. Clb2p does not accumulate in wild-type cells at the a-factor step but accumulates in wild-type cells arrested prior to anaphase with nocodazole (Amon et al. 1994). Clb2p accumulated in cells from strain 1904 arrested in mitosis by growth at the restrictive temperature (Figure 7). In contrast, Clb2p levels did not accumulate in cells of strain 1904 that were arrested in a factor and incubated at the restrictive temperature (Figure 7). Clb2p is undetectable in wild-type cells arrested in a factor (data not shown; Amon et al. 1994). However, a fraction of Clb2p was detected in cdc20 cells arrested at the a-factor step. We attribute this low level of Clb2p to cells (19%) that escaped the a factor block and continued on to arrest as large budded cells at the cdc20 step, a point in the cell cycle where APC activity is inhibited. We conclude that CDC20 function is not required to degrade Clb2p in cells arrested with a factor.

Although the bulk of Clb2p proteolysis occurs at the metaphase to anaphase transition, a fraction remains protected from proteolysis until the completion of Cdc15p-dependent processes in late anaphase (Irniger et al. 1995). Mutants compromised for APC function show increased sensitivity to galactose inducible CLB2 at the permissive temperature (Irniger et al. 1995). cdc16-123 and cdc23-1 mutants, grown at the permissive temperature, arrest in late anaphase with segregated chromosomes in response to high levels of Clb2p (Irniger et al. 1995). The interpretation is that even at the permissive temperature cdc16-123 and cdc23-1 mutants are limited for APC function, and the excess Clb2p accumulates to levels that prevent the exit from mitosis. We used this assay as an independent measure of CDC20 function in the APC. We determined that 30°C was the semipermissive temperature for growth of strain 1904 (bar1 cdc20 pGAL10::CLB2-3XHA) (data not shown). At the semipermissive temperature, cells are limited for CDC20 function and grow more slowly. If Cdc20p was required for the late anaphase proteolysis of Clb2p, then under semipermissive conditions, cells would be limited for Clb2p proteolysis, and excess Clb2p should cause cells to accumulate with a late nuclear division phenotype (segregated chromosomes). We grew cells from strain 1904 to midlogarithmic phase in SC-URA supplemented with 2% raffinose. Cells were then incubated at the semipermissive temperature in the presence of 2% galactose and analyzed after 4 hr. The cells were fixed in ethanol, and DNA morphology was analyzed by staining with DAPI. The majority of cells (55.6%) contained a single focus of DAPI stain located at the neck of the cell (medial nuclear division). We also observed unbudded cells with a single nucleus (18.5%) and large-budded cells with divided nuclei (25.6%). Therefore, unlike cdc16 and cdc23, cdc20 mutants did not arrest in late anaphase in response to elevated Clb2p levels (Irniger et al. 1995). These data suggest that Clb2p stability is unaffected in cdc20 cells arrested in a factor or cells that are exiting mitosis.

DISCUSSION

NDC10 and the spindle checkpoint: The spindle checkpoint prevents the onset of anaphase in yeast cells that have been treated with antimicrotubule drugs such as nocodazole and benomyl. The effects of these benzimida-zole drugs are complex. Microtubules are disassembled and the intracellular levels of tubulin dimers increase. Chromosomes become disassociated from the spindle and the spindle pole bodies collapse within the nucleus (Jacobs et al. 1988). In addition, microtubules may not be the only intracellular targets of these drugs as related benzimidazoles affect cytochrome p450 activity (Ortiz de Montellano 1995). It is formally possible that the benzimidazole drugs induce multiple lesions that can cause cell cycle arrest by acting through the MAD and BUB genes of the spindle checkpoint. There is ample evidence showing that impaired kinetochore function can trigger the spindle checkpoint (Wells 1996). In S. cerevisiae, mutations in centromere DNA,
multiple centromere-containing minichromosomes, dicentric chromosomes, and mutations in the gene encoding the kinetochore protein Ctf13p all cause a preanaphase arrest that is dependent on the spindle checkpoint (Neff and Burke 1992; Pangilinan and Spencer 1996; Wang and Burke 1995; Wells and Murray 1996). Which subset of lesions that are generated in response to nocodazole activate the spindle checkpoint?

We showed that ndc10 mutants are unable to arrest in the cell cycle in response to nocodazole. This suggests that the effect of nocodazole on the cell cycle is mediated exclusively through Ndc10p. Although it is possible that Ndc10p has multiple functions within the cell, the only one that has been characterized so far is in CBF3 function at the kinetochore (Goh and Kil martin 1993; Jiang et al. 1993; Sorger et al. 1995). We propose that nocodazole activates the spindle checkpoint because chromosomes become detached from the mitotic spindle. Supporting evidence for this conclusion comes from analysis of ctf13 mutants (Tavorina et al. 1997). Under some conditions, impaired Ctf13p function, which should also affect CBF3 activity, eliminates the spindle checkpoint. This suggests that the kinetochore, and proteins associated with CBF3, initiates the signaling that arrests the cell cycle in response to nocodazole.

The requirement for NDC10 in the spindle checkpoint can explain the unusual phenotype of ndc10 mutants. In the absence of Ndc10p function, the spindle elongates although the DNA is not attached and the result is that the chromosomes are asymmetrically distributed to the daughter cells. DNA replication continues in some of the cells and polyploid progeny are produced (Goh and Kilmartin 1993). These phenotypes differ from the preanaphase arrest that is induced in ctf13 mutants even though both NDC10 and CTF13 encode essential proteins of CBF3 (Lechner and Ortiz 1996). In vitro reconstruction of CBF3 suggests that ndc10 mutants lack CBF3 activity but ctf13 mutants retain a small amount (Sorger et al. 1995). Previous investigators have speculated that CBF3 activity is required for checkpoint signaling (Hyman and Sorger 1995; Sorger et al. 1995). Our data support this model. We propose that ctf13 mutants are defective in attaching microtubules to kinetochores but retain the capacity to signal, via the spindle checkpoint. The checkpoint signaling accounts for the preanaphase arrest (Pangilinan and Spencer 1996; Wang and Burke 1995). In contrast, ndc10 mutants can neither attach chromosomes nor signal for the arrest, which would account for the asymmetric segregation of chromosomes and the inability of ndc10 mutants to restrain DNA synthesis and arrest in the cell cycle (Goh and Kilmartin 1993).

It is possible that the different phenotypes displayed by mutants affecting CBF3 proteins are indicative of the strength of the mutations. Perhaps all of the available temperature-sensitive ctf13, skp1, and cep3 mutants retain some small amount of function in vivo. It is believed that a single unattached chromosome is capable of generating an inhibitory checkpoint signal (Rieder et al. 1995). If any mutant retains sufficient activity to assemble a partially functional kinetochore, then the single chromosome may be capable of inducing the checkpoint arrest. S. cerevisiae has 16 chromosomes, therefore the mutation would have to eliminate 95% (or more) of the activity to ensure that there was insufficient functional protein to construct a kinetochore. Perhaps only the ndc10 mutation is so effective. Unreplicated kinetochores lack checkpoint-signaling activity in ctf13 mutants (Tavorina et al. 1997). This suggests that the kinetochore that is assembled in the absence of DNA replication is different from the kinetochore that is assembled when DNA synthesis is completed normally. Perhaps CBF3 is less stable on an unreplicated kinetochore and therefore signaling is precluded. Alternatively, Ndc10p may play a dual role in kinetochore function. The protein may be required to assemble the functional kinetochore and may be the molecular site where chromosome attachments to the spindle are monitored and checkpoint signaling is initiated. A phosphoepitope with specific staining on misaligned or unattached kinetochores is further evidence that the kinetochore may be an important structure in the checkpoint-signaling pathway (Campbell and Gorbsky 1995; Nicklas et al. 1995). The Xenopus and human homologs of MAD2 and murine BUB1 localize to kinetochores (Chen et al. 1996; Li and Benezra 1996; Taylor and McKeeon 1997). If the sensor for the checkpoint localizes to the kinetochore, it is in the optimal position to detect errors in chromosome attachment and bipolar orientation.

**CDC20 and spindle function:** In yeast, perturbing the interaction of kinetochores with microtubules or destroying spindle structure with antimicrotubule drugs triggers the MAD- and BUB-dependent spindle checkpoints (Pangilinan and Spencer 1996; Wang and Burke 1995; Wells 1996). We were surprised that the mitotic arrest in CDC20-defective cells only required the BUB2 spindle checkpoint. However, independent studies also uncovered a difference between BUB2 and the other spindle checkpoint genes (Wang and Burke 1995; Pangilinan and Spencer 1996). In the absence of BUB2 function, cdc20-defective cells proceed in the cell cycle as assayed by spindle staining, budding morphology, and viability measurements. The effect was not immediate and required extended incubations at the restrictive temperature for cdc20 bub2::URA3 mutants to continue in the cell cycle. Kinetic analysis of spindle length distribution revealed that cells were able to delay at anaphase before undergoing an aberrant nuclear division and initiation of a new budding cycle in cdc20 bub2::URA3 mutants. Our results differ from previously published reports that failed to identify a role for BUB2 in the cdc20 arrest (Hyman et al. 1991). However, this is most likely due to the extended incubation periods required for
us to see the consequence of the bub2::URA3 mutation on the cdc20 arrest. These results are consistent with analysis of ctf13 bub2::LEU2 mutants that display marginal defects in chromosome segregation and cell cycle delay. After extended periods under restrictive conditions, viability decreases in ctf13 bub2::LEU2 mutants (Pangilinan and Spencer 1996). In an independent study, Wang and Burke (1995) did not detect a role for BU2B in the ctf13 arrest after a short (3 hr) exposure to restrictive conditions. We have examined viability after extended incubations in ctf13 bub2::URA3 cells and find a decrease in viability after 4 hr at the restrictive temperature (P. Tavorina, unpublished results). Our data suggest that bub2 mutants are able to initiate the inhibitory checkpoint pathway in response to impaired kinetochore function or a loss of CDC20 function but are deficient in maintaining the arrest. We conclude that by the strict genetic criterion of “relief of dependence,” CDC20 function is under Bu2B control (Hartwell and Weinert 1989). Our data are consistent with a distinct role for BU2B in signal maintenance as proposed by Pangilinan and Spencer (1996).

Relationship between CDC20 and the APC: Homologues of CDC20 have been identified in Drosophila melanogaster (fizzy), Schizosaccharomyces pombe (slp1), and humans (p55CDC), and all appear to be required for chromosome segregation (Dawson et al. 1995; Matsumoto 1997; Weinstein et al. 1994). Mutations in fizzy and in vivo depletion of p55CDC also result in a metaphase arrest with an enhancement of spindle microtubules (Dawson et al. 1993; Dawson et al. 1995; M. Kallio, D. J. Burke, J. Weinstein, and G. Gorbsky, unpublished results). Cyclin B, but not cyclin A, is stabilized in response to antimicrotubule drugs in Drosophila, Spisula, and Xenopus, suggesting that activating the spindle checkpoint results in cyclin B stability (Whiteleld et al. 1990; Hunt et al. 1992; Dawson et al. 1995; Minshull et al. 1994). However, mitotic cyclins A, B, and B3 are not degraded in fizzy mutant embryos, suggesting a specific role for the fizzy gene product in the destruction of cyclins at the metaphase to anaphase transition (Dawson et al. 1995; Sigrist et al. 1995). cdc20 mutants arrest before chromosome segregation with elevated levels of histone H1 kinase activity and Clb2p (Figure 3, Figure 6). Therefore, it has been suggested that CDC20, like fizzy, may play a role in regulating Clb2p levels during mitosis (Dawson et al. 1995; Yamamoto et al. 1996).

We found that Clb2p does not accumulate in cdc20 mutants arrested by α-factor. Clb2p destruction is active from anaphase onset until late G1 when Clnp accumulation inhibits the proteolysis of Clb2p (Amon et al. 1994). The essential components of the APC were identified based on their failure to degrade Clb2p in cells arrested in G1 by Clnp depletion (Irniger et al. 1995; Zachariae and Nasmyth 1996; Zachariae et al. 1996). Our results show that CDC20 function is not required for Clb2p proteolysis in α-factor arrested cells. This is consistent with the observation of Zachariae and Nasmyth (1996) that cells that are limited for CDC20 function are fully competent for Clb2p ubiquitination during an α-factor block. Furthermore, we extended this observation to show that Cdc20p is not involved in the late anaphase-specific function of the APC. We found that increased levels of Clb2p did not arrest cdc20 mutants (under semipermissive conditions) in anaphase, indicating that limiting CDC20 function has different consequences than either cdc16 or cdc23, which comprise APC function. These data suggest that CDC20 is distinct from CDC16 and CDC23 and is not an integral component of the APC.

One way to reconcile the differences between our data and the observations on Drosophila, fizzy is that a metaphase-anaphase-specific function of the APC requires CDC20. There is recent evidence to suggest that CDC20 regulates the metaphase to anaphase transition. High copy expression of CDC20 suppresses the temperature sensitivity of cdc28-1N, suggesting a link between CDC20 and the cell cycle machinery (Lim and Surana 1996; Yu et al. 1996). Increased expression of CDC20 can alleviate a RAD9-dependent arrest, suggesting that CDC20 can override the DNA damage checkpoint (Lim and Surana 1996). In addition, the S. pombe homologue of CDC20, slp1, was recently implicated in recovery from DNA damage-induced arrest (Matsumoto 1997).

CDC20 may also be able to affect cell cycle progression in cells arrested by the spindle checkpoint. A dominant allele of CDC20 (PAC5-1/CDC20-50) was recently identified in a screen for mutants that die in the absence of the spindle motor protein encoded by CIN8 (Geiser et al. 1997; E. Schott and M. A. Hoyt, personal communication). The arrest in response to deletion of CIN8 requires the MAD and BU2B checkpoint genes, suggesting that CDC20 may have a role in the checkpoint (Geiser et al. 1997). In fact, increased expression of CDC20 is able to override the spindle checkpoint-mediated arrest induced by nocodazole treatment or M Ps1 overexpression (E. Schott and M. A. Hoyt, personal communication). Furthermore, characterization of an S. cerevisiae homolog of CDC20, HCT1/CDH1 1 suggests that CDC20 may be a mitotic specific regulator of anaphase (Schwab et al. 1997; A. Amon, personal communication). HCT1/CDH1 is required for Clb2p destruction and Cdc20p may play a similar role in targeting the anaphase inhibitor, Pds1p, for destruction (Cohen-Fix et al. 1996; Schwab et al. 1997; E. Schott and M. A. Hoyt, personal communication; A. Amon, personal communication). Taken together, these data suggest that modulation of CDC20 activity may affect the cell cycle machinery. Cdc20p may be an activator of mitosis whose activity is inhibited in cells arrested by checkpoint control. Cdc20p may be required to restart the cell cycle during recovery from checkpoint-mediated arrest in addition to an essential function at the metaphase to anaphase transition. This would explain why cdc20 is epistatic to ndc10. If Cdc20p
plays a role in regulating anaphase progression, loss of CDC20 function may prevent continued cell cycle progression in ndc10 cells simply due to an inability to initiate anaphase events in the absence of CDC20 function.

By the genetic definition of checkpoint control (Hartwell and Weinert 1989), CDC20 function is under the control of the BU2B checkpoint. Cells defective for CDC20 are compromised in the cell division microtubule-mediated events of chromosome segregation and nuclear movements prior to anaphase (Palmer et al. 1989; Sethi et al. 1991). Sethi et al. (1991) proposed a role for Cdc20p in regulating microtubule stability during mitosis. Loss of CDC20 function has a dramatic effect on spindle structure as revealed by antitubulin staining and EM serial reconstruction (O'Toole et al. 1997; Sethi et al. 1991). Perhaps the lesion generated in cdc20-defective cells is a unique lesion recognized by BU2B. The lesion is not simply excess microtubules because the arrest in tub1 mutants with excess microtubules does not require BU2B. However, it remains a formal possibility that the molecular nature of the excess microtubules in cdc20 and tub1 mutants may differ and therefore constitute distinct lesions. A novel lesion in cdc20-defective cells seems less probable given the recent evidence supporting a role for CDC20 in the regulation of the metaphase to anaphase transition. The effect on microtubules in cdc20 mutants may be a secondary consequence of prolonged arrest at G2/M. One possibility is that the high levels of CDK activity at this point in the cell cycle may promote the continual polymerization of microtubules. Alternatively, altered APC function may promote microtubule assembly. A proportion of aberrant microtubule formation is also seen in some cdc23 mutants arrested at G2/M due to compromised APC function (M. Winey and T. Giddings, Jr., personal communication).

If CDC20 encodes a modulator of APC activity, our results raise intriguing possibilities for the role of BU2B in checkpoint maintenance. Sequence analysis has recently revealed that Bub2p is a member of a superfamily of proteins including the tre2 oncogene and two yeast genes encoding GTPase-activating proteins (Neuwald et al. 1997; Richardson and Zon 1995). A genetic interaction between the spg1+ encoded GTPase and the S. pombe homolog of BU2B, cdc16−, suggests that G-protein regulation may play a role in regulating late mitotic events (Schmidt et al. 1997). Furthermore, the S. cerevisiae homolog of spg1−, TEM1, has also been implicated in exit from mitosis (Shirayama et al. 1994). Bub2p may antagonize the activity of Cdc20p and maintain active Pds1p, the anaphase inhibitor. In the absence of Cdc20p and Bub2p, the cell may not maintain a mitotic arrest. The effect of Bub2p could be direct or could be effected through the action of HCT1, which has redundant Cdc20p activity. Further work is necessary to elucidate the interactions between these cell cycle regulators.

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