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 *BUB1*, *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.

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) (Lydall 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 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* (*cdc16*$^+$, *mad2*$^+$), *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 lesion monitored by the *MAD* and *BUB* spindle checkpoints 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 undivided nucleus at a stage indistinguishable from the *cdc20* arrest (Irniger *et al.* 1995; Lamb *et al.* 1995; Sethi *et al.* 1991). Recently, the *Drosophila melanogaster* homolog of *CDC20*, *fizzy*, has been implicated in cyclin degradation during mitosis (Dawson *et al.* 1995). A different interpretation for the arrest in *cdc20* 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 in *cdc20* mutants is independent of kinetochore function. We show that arrest is under control of the *BUB2* 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 of *CDC20* 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 after 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 *Eco*RV and *Sac*I 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 *Hin*dIII and *Xho*I 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^0 derivatives of strains H20C1B1 and MAY2099, respectively. rho^0 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-lacI::HIS3 lacO::LEU2*) and strain 1798 (*NDC10 GFP-lacI::HIS3 lacO::LEU*) were constructed by transforming strain 1749-33 (*ndc10-1 leu2 his3*) and W303 (*NDC10 leu2 his3)* with pAFS59 linearized with *Eco*RV. Leucine prototrophs were chosen and subsequently transformed with pAFS144 cut with *Nhe*I. Uracil 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 *HIS3* 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°. Samples were removed every 30 min and fixed in 3.7% formaldehyde for 15 min at room temperature. The cells were washed in 65 mm $NaPO_4$, 5 mm $MgCl_2$, mounted on slides, and GFP staining was visualized using standard FITC filters.

Indirect immunofluorescence and photomicroscopy: Antitubulin 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°. The monoclonal rat antitubulin antibody YOL 1/34 and secondary goat-anti-rat 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 mounted using the Slow-Fade-Light Antifade Kit (Molecular Probes, Inc., Eugene, OR). Microscopy and image acquisition were performed as described previously (Wang and Burke 1995).

Histone H1 kinase activity: 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, Inc., 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°, the restrictive temperature for *cdc20* mutants, for 2 hr to induce Clb2p synthesis. To examine Clb2p in *cdc20* cells at G2/M, cells from strain 1904 were grown to midlogarithmic phase in SC-URA supplemented with 2% raffinose. The culture was divided 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

Strain	Genotype	Source
405-1	*MAT***a** *ade2 his3 his7 ura3 cdc20-1*	Burke laboratory
5943-5	*MAT***a** *ade5 leu2 lys5 ura3 can1 cyh2*	Burke laboratory
H20C1B1	*MAT***a** *his7 ura1 cdc20-1*	L. Hartwell
796-8-2	*MAT***a** *his7 lys2 ndc10-169 cdc20-1*	This study
1738-4-2	*MAT***a** *cdc20-1 ndc10-1*	This study
492	*MAT***a** *leu2 lys5 ura3 trp1 ndc10-169*	M. Winey
1370	*MAT***a**/**a** *ade3/+ leu2/leu2 his7/his7 trp1/+ cyh2 cdc20-1/++ ura3/ura3*	This study
1370-3-1-3	*MAT***a** *ade3 leu2 his7 trp1 ura3 cyh2 cdc20-1 mad2::URA3*	This study
1370-3-1-4	*MAT*α *ura3 his7 leu2 cyh2 cdc20-1*	This study
1906	*MAT***a** *cdc20-1 his3 mad1::HIS3*	This study
1903	*MAT***a** *bar1 his7 ura3 cdc20-1*	This study
1904	*MAT***a** *bar1 his7 cdc20-1 ura3* p(*GAL10::CLB2* 3XHA)	This study
MAY2099	*MAT***a** *ura3 leu2 ade2 cdc20-1 bub2::URA3*	Hoyt *et al.* 1991
MAY2113	*MAT*α*his3 leu2 ura3 cdc20-1 bub3::LEU2*	Hoyt *et al.* 1991
MAY1786	*MAT***a** *ade2 his3 leu2 ura3 bub1-1 cdc20-1*	Hoyt *et al.* 1991
1907	*MAT***a** *his7 ura1 cdc20-1 rho*^{0}	This study
1908	*MAT***a** *ura3 leu2 ade2 cdc20-1 bub2::URA3 rho*^{0}	This study
JK418	*MAT***a** *ura3 leu2 trp1 ndc10-1*	Goh and Kilmartin 1993
1798	*MAT***a** *ade2 his3 leu2 ura3 GFP-Lac1-HIS3 LacO LEU2*	Burke laboratory
1749-33A	*MAT*α *ndc10-1 leu2 ura3 his3 lys2 hom3 can1 GFP-Lac1-HIS3 Lac0-LEU2*	This study
1760	*MAT***a** *his3 leu2 ura3 tub1::HIS3 tub3::TRP1* p*(tub1-730::LEU2)*	Schatz *et al.* 1988
1763	*MAT*α *his3 leu2 ura3 tub1::HIS3 tub3::TRP1* p*(tub1-758::LEU2)*	Schatz *et al.* 1988
1762	*MAT*α *his3 leu2 ura3 tub1::HIS3 tub3::TRPI* p*(tub1-741::LEU2)*	Schatz *et al.* 1988
1716-6-2	*MAT***a** *tub1::HIS3 tub3::TRP1 bub2::URA3 ade2 lys2* p*(tub1-730::LEU2)*	This study
1715-2-3	*MAT***a** *tub1::HIS3 tub3::TRP1 bub2::URA3 ade2 lys2* p*(tub1-75::LEU2)*	This study
1718-2-2	*MAT*α *tub1::HIS3 tub3::TRP1 bub2::URA3 ade2 lys2* p*(tub1-74::LEU2)*	This study

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 Clb2-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°. 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

Plasmid		Source
pKH149	*mad1::HIS3*	K. G. Hardwick and A. W. Murray
PRC10.1	*mad2::URA3*	R.-H. Chen and A. W. Murray
pWS945	*GAL10::CLB2 URA3* 3XHA	A. B. Futcher
pAFS59	*LacO* repeat in YIPlac128	A. F. Straight and A. W. Murray
pAFS144	*HIS3::GFP13-LacI-112*	A. F. Straight and A. W. Murray

was diluted 1000-fold in PBS-T with 10% milk and incubated overnight at 4°. 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 Kilmartin 1993; Jiang *et al.* 1993; Strunnikov *et al.* 1995). Cells complete mitosis, 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 Kilmartin 1993). Similiarly, 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.

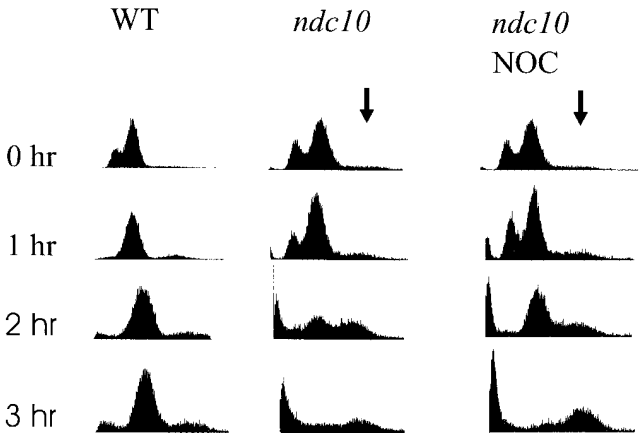

Figure 1.—DNA content of *ndc10* cells treated with nocodazole. Flow cytometry of cells from strain W303 (WT) and JK418 (*ndc10-1*) treated with 15 μg nocodazole per ml or 1% DMSO. Cells were grown to midlogarithmic phase, nocodazole or DMSO was added to the cultures and then the cells were shifted (0 hr) to the restrictive temperature for 3 hr. Cells were harvested each hour, fixed with ethanol, and stained with propidium iodide. The arrow indicates the population of cells displaying a 4N DNA content.

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-

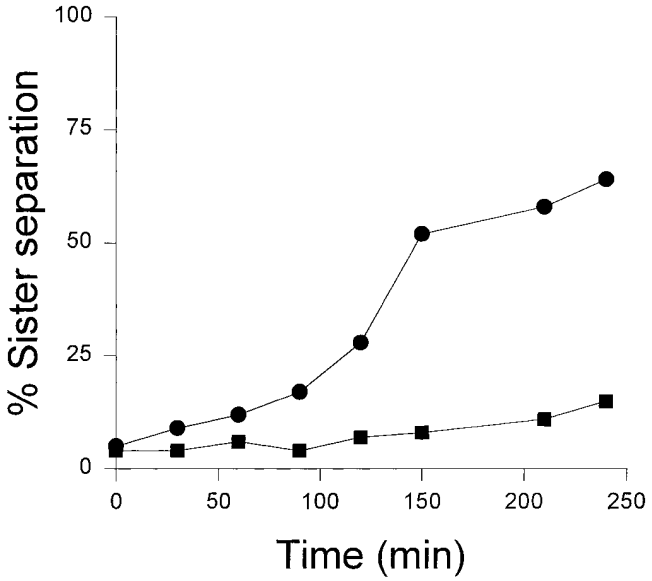

Figure 2.—Sister chromatid cohesion in *ndc10* cells. Cells from strains 1798 (*NDC10 GFP-lacI::HIS3 lacO::LEU2*) (■) and 1746-33A (*ndc10-1 GFP-lacI::HIS3 lacO::LEU2*) (●) were treated with 15 μg nocodazole per ml at the restrictive temperature. Two GFP-staining foci in the unseparated nuclei of large-budded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

tion to eliminate the kinetochore-dependent checkpoint-signaling pathway to ask whether the arrest in *cdc20* defective cells was dependent on kinetochore function. We constructed double mutants between *cdc20* and two different alleles of *ndc10* to ask if cells arrest in the absence of Ndc10p function. We tested the two different alleles of *ndc10* that vary in the degree of asymmetric division observed. *ndc10-169* mutants display less asymmetry in DNA distribution than *ndc10-1* mutants (Y. Wang and D. Burke, unpublished observations; Goh and Kilmartin 1993; Yoon and Carbon 1995). We examined the budding morphology and cylin-dependent kinase (CDK) activity in *ndc10* and *cdc20 ndc10* mutants. In *ndc10-169*, and *ndc10-1* mutants, there is an accumulation of unbudded cells, indicating that cells do not respond to the impaired kinetochore function and proceed through mitosis and cytokinesis (Table 3). In *cdc20-1*, *cdc20-1 ndc10-169*, and *cdc20-1 ndc10-1* cells, the majority of cells are large budded, indicating that *cdc20-1* is epistatic to *ndc10* (Table 3). We used CDK activity as an additional measurement of mitotic arrest. CDK acitvity reaches maximal levels in *cdc20* cells after growth for 4 hr under restrictive conditions. Therefore, we grew cells from strains 492 (*ndc10-169*), JK418 (*ndc10-1*), H20C1B1 (*cdc20-1*), 1738-4-2 (*cdc20-1 ndc10-1*), and 796-8-2 (*cdc20-1 ndc10-1*) for 4 hr at the restrictive temperature of 37° and measured CDK activity as the ability to phosphorylate histone H1 *in vitro*. CDK activity was low in cells lacking *NDC10* function. However, CDK activity was elevated to mitotic levels in cells from strains H20C1B1 (*cdc20-1*), 796-8-2 (*cdc20-1 ndc10-169*), and 1738-4-2 (*cdc20-1 ndc10-1*), indicating that the double mutants arrest at mitosis (Figure 3). These results suggest that the arrest in *cdc20*-defective cells does not require kinetochore-dependent checkpoint signaling.

TABLE 3

Budding morphology in *cdc20 ndc10* mutants

Genotype	Treatment (4-hr)	Percent budded morphology			
		U[a]	SB[b]	LB[c]	MB[d]
CDC20 NDC10	36°	34	38	28	0
cdc20-1	36°	23	4	69	4
ndc10-1	36°	54	9	35	2
cdc20-1 ndc10-1	36°	9	2	86	3
ndc10-169	36°	41	18	39	2
cdc20-1 ndc10-169	36°	26	1	67	6

[a] U, unbudded.
[b] SB, small budded.
[c] LB, large budded.
[d] MB, multibudded.

***cdc20* arrest requires *BUB2* but not other spindle checkpoint genes:** The cell cycle arrest in *cdc20* mutants may require spindle checkpoint genes and still be independent of kinetochore function. This seems likely given that spindle assembly is altered in *cdc20* mutants that are arrested at the restrictive temperature (Sethi *et al.* 1991; O'Toole *et al.* 1997). To determine if the cell cycle arrest in *cdc20* mutants required the spindle checkpoint genes, we analyzed spindle morphology in checkpoint double mutants. Spindle structure and cell morphology are unaffected at high temperatures in *mad* and *bub* single mutants (Pangilinan and Spencer 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-

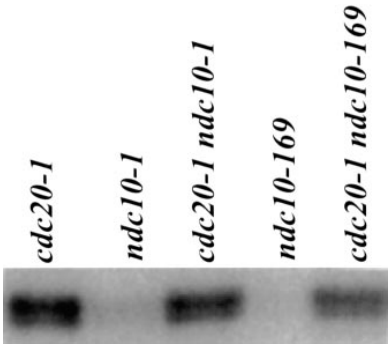

Figure 3.—Effect of *cdc20* on cells deprived of *NDC10* function. Histone H1 kinase activity in cells from strain H20C1B1 (*cdc20-1*), 492 (*ndc10-169*), 796-8-2 (*cdc20-1 ndc10-169*), JK418 (*ndc10-1*), and 1738-4-2 (*cdc20-1 ndc10-1*) after a 4-hr incubation at the restrictive temperature.

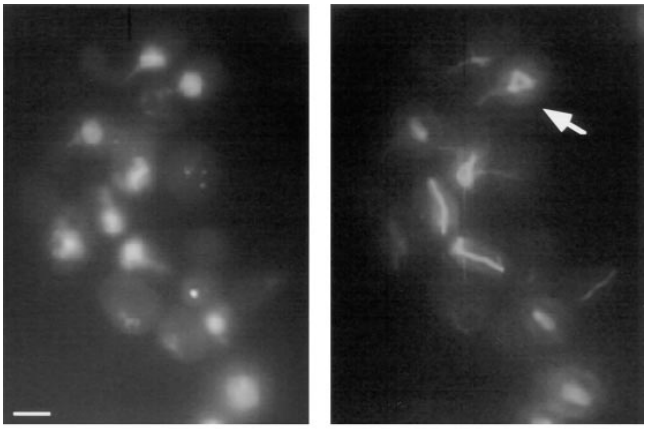

Figure 4.—Spindle morphology in *cdc20 bub2::URA3* mutants. Cells from strain 1908 (*cdc20 bub2)* were grown for 4 hr at the restrictive temperature. Cells were fixed in formaldehyde and processed for antitubulin staining and DAPI staining. The arrow indicates an example of a multipolar spindle. Bar, 5 μm.

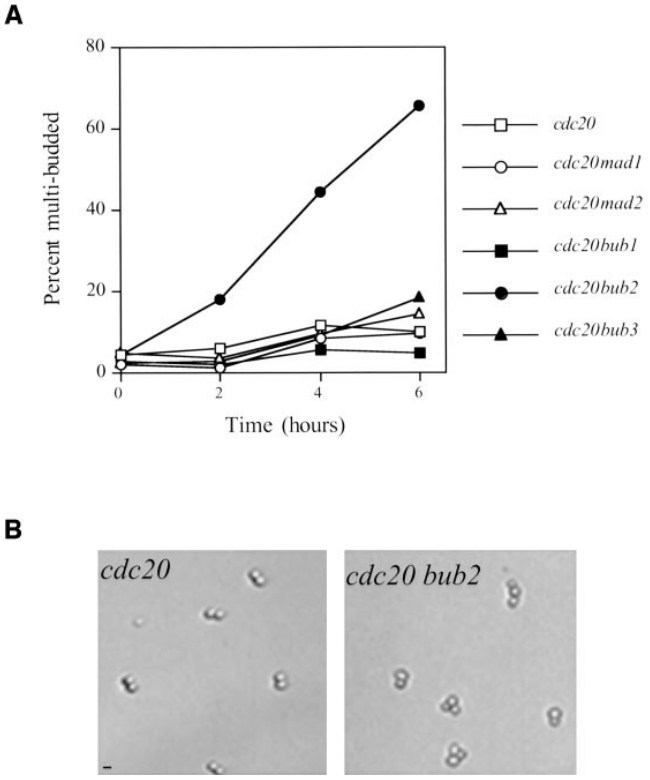

Figure 5.—*cdc20* arrest requires the *BUB2* spindle checkpoint. (A) Percentage of cells from strains MAY1787 (*cdc20 bub1*), 1908 (*cdc20 bub2*), MAY2113 (*cdc20 bub3*), 1906 (*cdc20 mad1*), 1370-3-1-3 (*cdc20 mad2*), and 1370-3-1-4 (*cdc20)* displaying multiple buds (≥3) after shifting to the restrictive 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.

nofluoresence 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 (Sethi *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 strain 1370-3-1-4 (*cdc20)* to midlogarithmic phase. Cells were shifted to the restrictive temperature and budding morphology examined each hour over a 6-hr period. These data (Figure 5) indicate that *cdc20 bub2* cells initiate a new budding cycle, while *cdc20*, *cdc20 mad*, *cdc20 bub1*, and *cdc20 bub3* cells remain as large budded cells during extended times at the restrictive temperature. After ~2 hr at the restrictive temperature, *cdc20 bub2* cells begin to bypass the *cdc20* block and proceed in the cell cycle. Initiation of a new budding cycle requires B-type cyclin proteolysis and activation of G1-cyclin (CLN) activity in the subsequent cell cycle (Amon *et al.* 1994). We confirmed that CDK activity was not maintained at mitotic levels in *cdc20 bub2* cells, suggesting that late events in the cell cycle are executed (data not shown). We conclude that *bub2::URA3* is able to relieve the dependency of late cell cycle events on the function of *CDC20*.

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

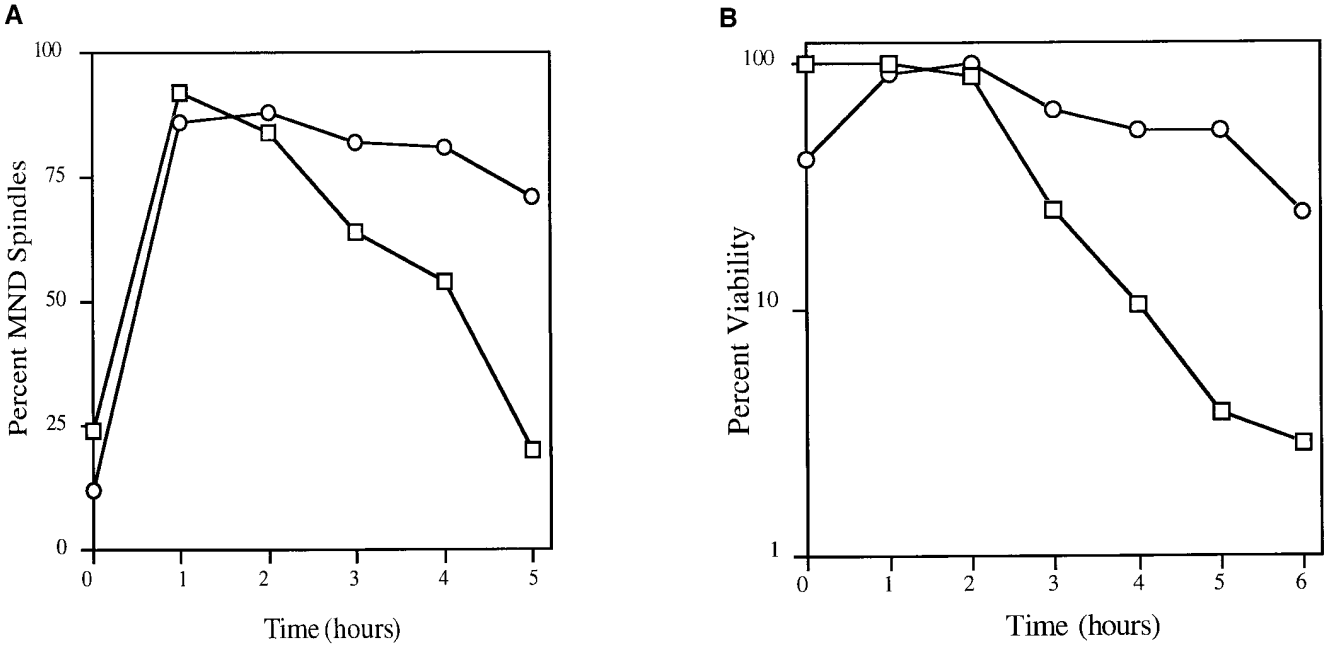

Figure 6.—*cdc20 bub2::URA3* mutants cannot maintain a mitotic arrest. (A) Percentage of cells displaying medial nuclear division (MND) spindles in cells incubated at 36°. Cells from strain 1908 (*cdc20 bub2*) and strain H20C1B1 (*cdc20*) were released from α-factor arrest and grown at the restrictive 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°), 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°, 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°). 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 α-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 α 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 α-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 α factor and incubated at the restrictive temperature (Figure 7). Clb2p is undetectable in wild-type cells arrested in α factor (data not shown; Amon *et al.* 1994). However, a fraction of Clb2p was detected in *cdc20* cells arrested at the α factor step. We attribute this low level of Clb2p to cells (19%) that escaped the α 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 α 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° 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 α factor or cells that are exiting mitosis.

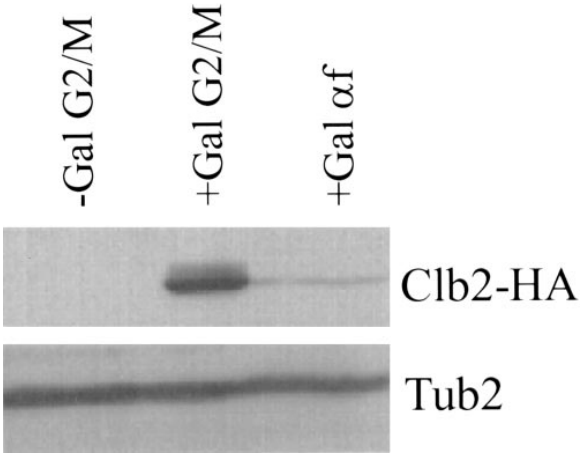

Figure 7.—Clb2p levels in *cdc20* cells at the α-factor step. Total protein extracts of cells from strain 1904 (*bar1 cdc20*) harboring plasmid pWS945 (*pGAL10::CLB2* 3X HA) were analyzed by immunoblotting with anti-HA or with anti-Tub2p antibodies. Cells were incubated at 36° for 2 hr either in the presence (αf) or absence (G/M) of α factor and either with galactose induction (+Gal) or without induction (−Gal) before harvesting.

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 benzimidazole 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 Kilmartin 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 (Tavormina *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 (Tavormina *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 McKeon 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 (Hoyt *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 *BUB2* 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. Tavormina, 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 *BUB2* control (Hartwell and Weinert 1989). Our data are consistent with a distinct role for *BUB2* 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 (Whitfield *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 Clbp 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. Clbp destruction is active from anaphase onset until late G1 when Clnp accumulation inhibits the proteolysis of Clbp (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 *BUB* 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 *MPS1* overexpression (E. Schott and M. A. Hoyt, personal communication). Furthermore, characterization of an *S. cerevisiae* homolog of *CDC20*, *HCT1/CDH1* 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 *BUB2* 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 *BUB2*. The lesion is not simply excess microtubules because the arrest in *tub1* mutants with excess microtubules does not require *BUB2*. 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 *BUB2* in checkpoint maintenance. Sequence analysis has recently revealed that Bub2p is a member of a superfamily of proteins including the *tre-2* oncogene and two yeast genes encoding GTPase-activating proteins (Neuwald 1997; Richardson and Zon 1995). A genetic interaction between the $spg1^+$ encoded GTPase and the *S. pombe* homolog of *BUB2*, $cdc16^+$, 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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