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Changes in the Localization of the *Saccharomyces cerevisiae* Anaphase-Promoting Complex Upon Microtubule Depolymerization and Spindle Checkpoint Activation

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ABSTRACT

The anaphase-promoting complex/cyclosome (APC/C) is an E3 ubiquitin ligase in the ubiquitin-mediated proteolysis pathway (UMP). To understand how the APC/C was targeted to its substrates, we performed a detailed analysis of one of the APC/C components, Cdc23p. In live cells, Cdc23-GFP localized to punctate nuclear spots surrounded by homogenous nuclear signal throughout the cell cycle. These punctate spots colocalized with two outer kinetochore proteins, Slk19p and Okp1p, but not with the spindle pole body protein, Spc42p. In late anaphase, the Cdc23-GFP was also visualized along the length of the mitotic spindle. We hypothesized that spindle checkpoint activation may affect the APC/C nuclear spot localization. Localization of Cdc23-GFP was disrupted upon nocodazole treatment in the kinetochore mutant *okp1-5* and in the *cdc20-1* mutant. Cdc23-GFP nuclear spot localization was not affected in the *ndc10-1* mutant, which is defective in spindle checkpoint function. Additional studies using a *mad2*Δ strain revealed a microtubule dependency of Cdc23-GFP spot localization, whether or not the checkpoint response was activated. On the basis of these data, we conclude that Cdc23p localization was dependent on microtubules and was affected by specific types of kinetochore disruption.

DURING mitosis, ubiquitin-mediated proteolysis (UMP) controls two distinct events: anaphase and exit from mitosis (Holloway *et al.* 1993; Surana *et al.* 1993). Mitotic proteolysis is regulated by the anaphase-promoting complex/cyclosome (APC/C), an E3 enzyme in the UMP pathway (Morgan 1999; Weissman 2001; Peters 2002). The APC/C consists of 8–13 subunits, depending on the organism (Peters *et al.* 1996; Zachariae *et al.* 1996, 1998; Yu *et al.* 1998; Yoon *et al.* 2002; Hall *et al.* 2003; Passmore *et al.* 2003). In this work, we investigate the localization of the APC/C in *Saccharomyces cerevisiae* primarily using the APC/C subunit Cdc23p. *CDC23* is an essential gene and encodes an essential APC/C component that binds to other APC/C core components, Cdc16p and Cdc27p, as well as to the Clb2p N terminus (Sikorski *et al.* 1990, 1993; Lamb *et al.* 1994; Meyn *et al.* 2002).

APC/C activity is regulated by two specificity factors, Cdc20p and Hct1p/Cdh1p (Harper *et al.* 2002; Irniger 2002). In most organisms, including *S. cerevisiae* and humans, Cdc20p interacts with the APC/C to ubiquitinate the metaphase-anaphase inhibitor Pds1p, allowing sister chromatid separation and passage into anaphase, while Hct1p regulates APC/C activity during mitotic exit and G_1 (Cohen-Fix *et al.* 1996; Yamamoto *et al.* 1996a,b; Schwab *et al.* 1997; Visintin *et al.* 1997; Shirayama *et al.* 1998, 1999). In *S. cerevisiae*, Cdc20p-dependent but not Hct1p-dependent APC/C activity is impaired upon mutation of Cdc28p consensus phosphorylation sites in Cdc23p and two other major APC/C subunits (Rudner and Murray 2000).

The spindle checkpoint, a spindle damage surveillance system, blocks cell cycle progression by inhibiting the APC/C (Musacchio and Hardwick 2002; Yu 2002; Cleveland *et al.* 2003). Spindle checkpoint components include the kinase Mps1p, and Mad1-3p and Bub1-3p, first discovered in budding yeast as mutants that failed to respond to microtubule-depolymerizing drugs (Hoyt *et al.* 1991; Li and Murray 1991; Weiss and Winey 1996). Cdc20p is known to interact with the spindle checkpoint pathway members Mad1p, Mad2p, Mad3p, and Bub3p (Hwang *et al.* 1998; Hardwick *et al.* 2000). In budding yeast, the APC/C and Mad2p binding sites of Cdc20p are overlapping, suggesting a mutually exclusive relationship (Zhang and Lees 2001). On the basis of extensive genetic and biochemical analysis, spindle checkpoint inhibition of the APC/C is mediated by Cdc20p.

In contrast, little is known about the spatial mechanism of APC/C inhibition by the spindle checkpoint pathway. In yeast and humans, several members of the spindle checkpoint pathway are at least transiently localized to the kinetochore (Lew and Burke 2003; McAinsh *et al.* 2003). In humans, these proteins include Cdc20, hBub1, hBubR1, Mad2, and Mad1 (Li and Benezra 1996; Taylor and McKeon 1997; Jablonski *et al.* 1998; Kallio

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et al. 1998). Recent studies in yeast have found similar results with Mps1p, Bub1p, Bub3p, Mad1p, and Mad2p (Castillo *et al.* 2002; Iouk *et al.* 2002; Kerscher *et al.* 2003; Kitagawa *et al.* 2003; Gillett *et al.* 2004). We hypothesize that the APC/C has a dynamic localization during mitosis and the rest of the cell cycle. After observing APC/C localization in budding yeast during a normal cell cycle, we can then determine if its localization changes in response to spindle checkpoint activation or other perturbations.

Limited information about APC/C localization is available from studies performed in other organisms. Subunits of the mammalian APC/C have been localized to the kinetochore, centrosome, and mitotic spindle (Tugendreich *et al.* 1995; Jorgensen *et al.* 1998; Kurasawa and Todokoro 1999; Topper *et al.* 2002). Drosophila APC/C is found on the mitotic spindle, and the green fluorescent protein (GFP)-tagged APC/C subunit Cdc27p has been localized to mitotic chromosomes (Huang and Raff 1999, 2002). In *S. cerevisiae*, where immunolocalization is more difficult to resolve, the APC/C subunit Cdc23p has thus far been described as primarily nuclear (Sikorski *et al.* 1993; Jacquenoud *et al.* 2002; Huh *et al.* 2003). On the basis of the localization of known APC/C substrates to the mitotic apparatus (such as Kip1p or Ase1p), one might predict that the *S. cerevisiae* APC/C would also be found on the mitotic spindle, the spindle poles, and/or the kinetochores (Juang *et al.* 1997; Gordon and Roof 2001).

In this work, we describe cell-cycle-dependent changes in the localization of the APC/C subunit Cdc23p. In addition, we focus on the relationship between the spindle checkpoint status and APC/C localization. Our comprehensive analysis not only confirms a similar localization pattern for the APC/C in *S. cerevisiae* and mammalian cells, but also extends these studies by identifying factors, such as microtubule depolymerization and defective kinetochore structure, that can affect this localization.

MATERIALS AND METHODS

Strain construction: The C terminus of *CDC23* and *APC9* were tagged with green fluorescent protein (*GFP-kanMX6*) using a PCR-based method described previously (Longtine *et al.* 1998). We used a GFP construct containing the mutations S65T and F64L, made by I-ching Yu in the Pringle lab (gift of E. Bi) to generate a fusion of GFP to the gene of interest driven by the endogenous gene's promoter. Cyan fluorescent protein (CFP; pDH3) and yellow fluorescent protein (YFP; pDH5) plasmids were obtained from the Yeast Resource Center (http://depts.washington.edu/yeastrc/index.html). The GFP lumenal marker for the endoplasmic reticulum (pADH-ERGFP) was obtained from the Rose lab (Gammie and Rose 2002). In addition, the *CFP-TUB1* (*URA3*) plasmid was obtained from the Reed lab (Jensen *et al.* 2001). The *mad2::URA3* deletion construct (pRC10.1) was a gift of A. Murray (Chen *et al.* 1999). Strains used in this study are listed in Table 1. For all primers used for construct design, see Table 2.

Cdc23-GFP and Apc9-GFP strains were tested for viability by serial dilution from 23°–37° (data not shown). No loss of viability or growth delay was seen with any strains in the YSH1071 (YEF473) background. In Cdc23-GFP tagged strains in the YSH21 background, a growth delay was observed above 35° after repeated serial dilutions. Cells were fixed with ethanol, stained with 4′,6-diamidino-2-phenylindole (DAPI), and scored microscopically to determine the cell cycle distribution by approximating bud size and nuclear position. This type of cell cycle analysis of Cdc23-GFP and Apc9-GFP strains after a 4-hr temperature shift to 37° (typical experiment length) indicated that no cell cycle stage delay was observed and that GFP expression was comparable to that of other strains (data not shown). All kinetochore strains were tested by serial dilution after CDC23-GFP tagging. No changes in their temperature-sensitive growth pattern were observed after tagging (data not shown).

Imaging and analysis: To optimize the GFP signal, in particular the intensity of the nuclear signal, samples were taken from log-phase, well-aerated liquid YPD cultures (spun down and resuspended in minimal SD media supplemented with necessary amino acids for observations). Cultures were grown at 23° unless indicated otherwise. Supplemental adenine was added to cultures of *ade2*$^-$ strains.

Unfixed and DAPI-stained cells were observed on a microscope (Eclipse E800, Nikon, Tokyo) connected to a cooled, high-resolution charge-coupled device (CCD) camera (ORCA model C4742-95, Hammatsu Photogenics, Bridgewater, NJ). Images were analyzed using Image-Pro (Phase 3) Imaging software (Media Cybernetics, Silver Spring, MD). Differential interference contrast (DIC) images (exposure time, 0.6 sec), DAPI images (exposure time, 2 sec), and GFP images (exposure time, 5 sec) were taken as indicated. Contrast was enhanced using this Phase 3 Imaging program and Adobe Photoshop 4.0 (Adobe Systems, San Jose, CA).

APC/C localization in various mutant strains, as well as CFP/YFP colocalization studies, was conducted using a Leica DMRA2 microscope connected to a cooled, high-resolution CCD camera (ORCA extended range camera, Hammatsu Photogenics). Images were analyzed using OpenLab version 3.0.2 (Improvision). Filter sets for this microscope included cyan GFP v2 (D436/20 D480/40 455DCLP), yellow GFP BP (10C/Topaz—HQ500/20 HQ535/30 Q515LP), and a GFP filter set (E450/50D 480BP 510/50). On this microscope, exposure time for Cdc23-GFP was 4 sec, with 1× binning, while exposure time for Cdc23-YFP was 1.5 sec with 2× binning. Okp1-CFP and Slk19-CFP images were taken at 1 and 0.6 sec, respectively, with 2× binning and 20% gain. Spc42-CFP settings were the same as those for Slk19-CFP. Cdc20-GFP settings were the same as for Cdc23-YFP. Additional images were also collected using the DeltaVision Microscopy System.

For colocalization studies using CFP-tagged cellular markers and Cdc23-YFP, cells were put into three categories. For complete colocalization, the spindle pole body or kinetochore marker signal/signals had to overlap with the Cdc23p spot signal/signals. In the case of partial colocalization, this usually occurred when one spot signal was visible for one marker, but two were present for the second marker. For example, in some cases, two kinetochore-CFP spots were visible, but only one Cdc23-YFP spot was seen. Therefore, the overlap of the single Cdc23p spot with one of the kinetochore markers was defined as partial colocalization. Finally, no overlap of the spindle pole body or kinetochore signal with the Cdc23-YFP nuclear spot signal was defined as "no colocalization."

Indirect immunofluorescence was conducted to visualize microtubules in the *okp1-5* mutant. We followed a standard protocol for this analysis, using the YOL1/34 rat anti-α-tubulin primary antibody and a FITC anti-rat secondary antibody (gift of S. Clark; Pringle *et al.* 1991).

Cell synchronization and treatment of temperature-sensitive strains: For nocodazole synchronization, asynchronous cultures

TABLE 1

Strains used in this study

Strain (alias in parentheses)	Relevant genotype	Source
YSH1071 (YEF473)	**a**/α *his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3*	Bi and Pringle (1996)
YSH1072	As YSH1071 except *CDC23:GFP/CDC23*	This study
YSH1190	As YSH1071 except *CDC23:GFP/CDC23:GFP*	This study
YSH1558	As YSH1071 except *NDC10:GFP/NDC10*	This study
YSH1527	As YSH1071 except *CDC20:GFP/CDC20*	This study
YSH1175	As YSH1071 except *APC9:GFP/APC9*	This study
YSH1554	As YSH1071 except *OKP1:GFP/OKP1*	This study
YSH1152	As YSH1071 except pADH-ERGFP [ss-HDEL-*GFP* (*TRP1*)]	This study
YSH1197	As YSH1071 except p*GFP*-C-FUS (GFP alone)	This study
YSH21 (YPH499)	*MAT***a** *ura3-52 leu2Δ1 his3Δ200 trp1Δ63 lys2Δ800 ade2-101*	P. Hieter
YSH1750 (MAY589)	*MAT***a** *ura3-52 leu2-3,112 his3Δ200 ade2-101*	A. Hoyt
YSH1332	As YSH21 except *CDC23:GFP*	This study
YSH1751	As YSH1750 except *CDC23:GFP*	This study
YSH1753	As YSH1751 except *mad2Δ*	This study
YSH1161	As YSH21 except *APC9:GFP*	This study
YSH1503	As YSH21 except *CDC23:YFP*	This study
YSH1334	As YSH1503 except p*CFP-TUB1* (*URA3*)	This study
YSH1154 (FLY694)	*MAT*α *his3 ura3 leu2 trp1 SPC42:CFP*	Luca *et al.* (2001)
YSH36 (YPH500)	As YSH21 except *MAT*α	P. Hieter
YSH1509	As YSH36 except *SLK19:CFP*	This study
YSH1551	As YSH36 except *OKP1:CFP*	This study
YSH1506	**a**/α *his3/his3 ura3/ura3 leu2/leu2 trp/trp SPC42:CFP/SPC42 CDC23:YFP/CDC23*	This study
YSH1511	**a**/α *his3/his3 ura3/ura3 leu2/leu2 trp/trp SLK19:CFP/SLK19 CDC23:YFP/CDC23*	This study
YSH1560	**a**/α *his3/his3 ura3/ura3 leu2/leu2 trp/trp OKP1:CFP/OKP1 CDC23:YFP/CDC23*	This study
YSH1599	**a**/α *his3/his3 ura3/ura3 leu2/leu2 trp/trp OKP1:CFP/OKP1 SLK19:YFP/SLK19*	This study
YSH1530 (YJL158)	*MAT***a** *ura3-52 leu2Δ1 his3Δ200 trp1Δ63 lys2-801 ade2-101 okp1-5::TRP1 cyh2*	Ortiz *et al.* (1999); Chen *et al.* (2000)
YSH1565	As YSH1530 except *CDC23:GFP*	This study
YSH1582	As YSH1530 except *SLK19:GFP*	This study
YSH1562	As YSH1530 except *mad2Δ*	This study
YSH1563	As YSH1530 except *mad2Δ*	This study
YSH1534 (JVK418)	*leu2 lys2 ura3-52 trp1 ndc10-1*	He *et al.* (2001)
YSH1576	As YSH1534 except *CDC23:GFP*	This study
YSH1186	*MAT***a** *ura3-52 leu2,3-112 trp1 can1 ade2 his3-11,15*	D. Roof
YSH1575	As YSH1186 except *CDC23:GFP*	This study
YSH1200 (KH 153)	As YSH1186 except pGAL-*MPS1:URA3*	Hardwick *et al.* (1996)
YSH1361	As YSH1200 except *CDC23:GFP*	This study
AR1979	As YSH1200 except *CDC27-5A:KanR CDC16-6A:TRP1 CDC23-A-HA*	Rudner and Murray (2000)
YSH1517	As AR1979 except *CDC23-A-HA:YFP*	This study
YSH382 (MAY1715)	*MAT***a** *his3 leu2 ura3 ade2 can1 cdc20-1*	Schott and Hoyt (1998)
YSH1348	As YSH382 except *CDC23:GFP*	This study
YSH1095	*MAT***a** *leu2-3 ura 3 trp1-1 cdc15-2*	Visintin *et al.* (1998)
YSH1180	As YSH1095 except *CDC23:GFP*	This study
YSH1583	As YSH1200 except *NUF2:GFP*	This study
YSH1585	As YSH1200 except *OKP1:GFP*	This study
YSH1587	As YSH382 except *OKP1:GFP*	This study
YSH1588	As YSH382 except *NUF2:GFP*	This study
YSH1590	As YSH1534 except *OKP1:GFP*	This study
YSH1592	As YSH1534 except *NUF2:GFP*	This study

TABLE 2

Primers used for construct design

Tagging and diagnostic primers	Sequence
CDC23F2/2 (tagging)	GAA GAG GCT CGT ATG CTG GCT CGG GAG TGC AGA AGG CAT ATG CGG ATC CCC GGG TTA ATT AA
CDC23R1/2	TGG TCA CTT TAA ATA GCT ATA TAG CCT GGC ATT TAT AAA AGA ATT CGA GCT CGT TTA AAC
CDC23F2-G	ATT GAA GAG GCT CGT ATG CTG GCT CGG GAG TGC AGA AGG CAT ATG GGA GGA GGA GGA GGA CGG ATC CCC GGG TTA ATT AA
CDC23R1-G	CGG TTC ATC CTT CTG CCA TGG TCA CTT TAA ATA GCT ATA TAG CCT GGC ATT TAT AAA AGA ATT CGA GCT CGT TTA AAC
CDC20F2-G	ATT CAT ACA AGG AGG CCC TCT AGT ACC AGC CAA TAT TTG ATC AGG GGA GGA GGA GGA GGA CGG ATC CCC GGG TTA ATT AA
CDC20R1-G	CTT CTT GAA TAC ATT TAT GTT TAT ACA TTA TGT ATG CGT GTA TGG AAA TTT CAT GAA TTC GAG CTC GTT TAA AC
APC9F2	AAG TGC AGA AGA CAC TAT AGC AAC GAG GAT AGT TCG TTT CTC CGG ATC CCC GGG TTA ATT AA
APC9R1	GTA TAT ATA TAT ATG TGT GTA TAT ACA TGT ATG TCT GTA TAG GAA TTC GAG CTC GTT TAA AC
NDC10F2-G	GAC CAT CAA AAT TCA TTT GAT GGT CTG TTA GTA TAT CTA TCT AAC GGA GGA GGA GGA GGA CGG ATC CCC GGG TTA ATT AA
NDC10R1-G	ACA GCA ACC GTT GAT ATT CCG GCA GCC TTT TTC TCA TAG TAA ACA TCA TTA TAT TAT AAA GAA TTC GAG CTC GTT TAA AC
NUF2F2-G	GGT CAT ATT AAT AAA TAC ATG AAT GAA ATG CTC GAA TAT ATG CAA GGA GGA GGA GGA GGA CGG ATC CCC GGG TTA ATT AA
NUF2R1-G	AGG TAC GTA TAA GAT TAA GAA ACA GCC ACG AAA GAA ACA ACA GTA AAT TCC GAA TTC GAG CTC GTT TAA AC
SLK19F2-G	GAA GAA AAG CAG GAG TTA CTC AAG TTG TTA GAA AAT GAA AAA AAA GGA GGA GGA GGA GGA CGG ATC CCC GGG TTA ATT AA
SLK19R1-G	GGA AAC AAT ATC AAA TAT TCG ATA CGA ATT TGT ATA TTT ACG GTA TAT CCT TAA TAG AAT TCG AGC TCG TTT AAA C
OKP1F2-G	CCG CAC CAT GAG TCG CAC CAA GAT AAG ACC GAA GAA GAT ATA CAC GGA GGA GGA GGA GGA CGG ATC CCC GGG TTA ATT AA
OKP1R1-G	GAG GCT CGT TGG CGG AGA AGT TAC GTA ACC CGG ATG CTA ATA GTG CAT GAT TAT ATA TCA GAA TTC GAG CTC GTT TAA AC
GFP-721R (diagnostic)	TTT GTA TAG TTC ATC CAT GCC ATG T
GFP-481F	CAG ACA AAC AAA AGA ATG GAA TCA AAG
CDC23-1384F	GCG AAT TCC CTC GAG ACT TCA AAG
CDC23-1561F	TGC TAC AAA AGA TCC A
APC9-380F	CAC CGT TTT GTG AGC
APC9-1298R	GTC TTC GCT TAT ATA CAG AAG AAG AAA C
CDC20-1730F	ATG AAA CAA AAT TCA AAG TTG
NDC10-2700F	GAA GCA AAG AGC TAT ACT GAC GAA
NUF2-1394F	TCA AGA GGA ACT GAT CAG TGG
SLK19-2454F	GCA GAA GAT TTG TAT ATC CA
OKP1-1121F	ATG GCT TAG AAT CTA CTA ATG GA

Primers ending with "G" have five glycines between the gene and GFP; no difference in GFP signal was seen with glycines added for CDC23-GFP tagging.

were treated with 15 μg/ml of the drug (M1404, Sigma, St. Louis) for 4 hr at 23°. At this concentration, typically 80% of the cells were arrested at metaphase. For temperature-sensitive strains, cells were grown overnight at 23° and then shifted to 37° for 4 hr. Cells were determined to be arrested when the percentage of cells at the arrest point matched previously published reports for that strain. Samples from representative experiments were fixed in 100% ethanol at this time to analyze the nuclear position by DAPI staining (1 μg/ml) to verify the arrest (data not shown).

RESULTS

Localization of the APC/C in *S. cerevisiae*: We used a GFP-tagging approach to determine the localization pattern of the APC/C throughout the cell cycle. We selected one essential (*CDC23*) and one nonessential (*APC9*) subunit of the APC/C to initiate our studies on APC/C localization. The gene encoding the GFP was integrated at the C terminus of the endogenous locus of either

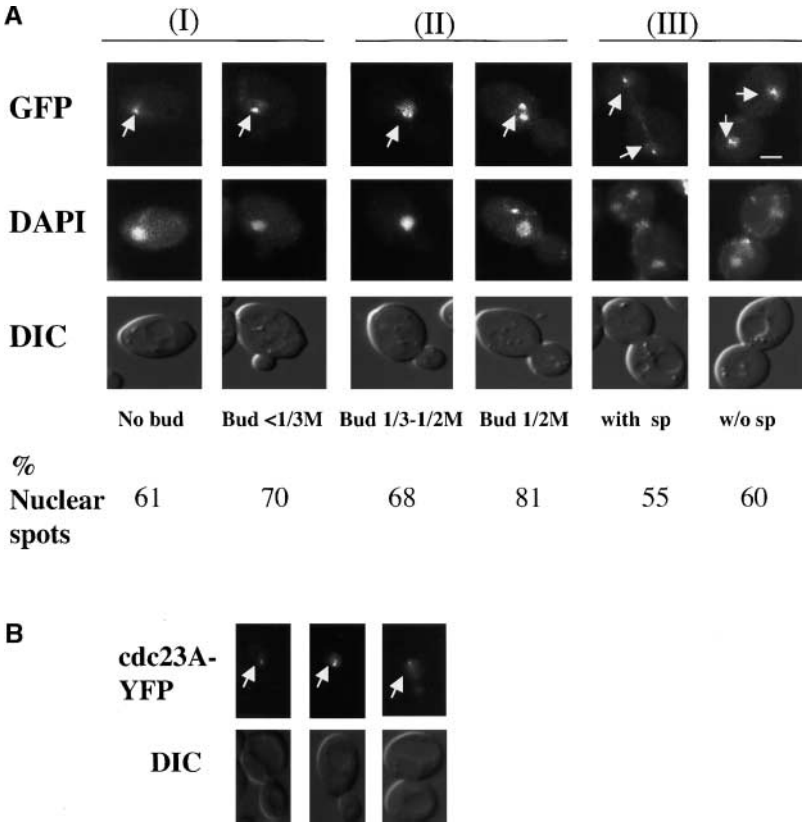

FIGURE 1.—Cdc23-GFP is localized to the nucleus and nuclear spots throughout the cell cycle and moves onto the mitotic spindle in anaphase. (A) Examples of the Cdc23-GFP signal (YSH1072, YSH1190) at each stage of the cell cycle, with a corresponding DIC and DAPI image for each GFP image. Arrows indicate the Cdc23-GFP nuclear spot signal ($n = 222$). Cells were divided into three categories as judged by bud size and nuclear position. Category I contains unbudded cells (none) and cells with bud less than one-third the size of the mother bud (<1/3M). Category II contains cells with a bud one-third to one-half the size of the mother bud. Finally, category III includes cells in anaphase/telophase either with or without a spindle signal present. The percentage of cells with a nuclear spot signal, in addition to the nuclear signal, is shown for each stage. (B) YFP and DIC images of an APC/C phosphorylation mutant, *cdc16-6A cdc27-5A cdc23A:YFP* (YSH1517). Bar, 3 μm.

CDC23 or *APC9*. The Cdc23-GFP and Apc9-GFP fusion proteins were visualized in live cells using fluorescence microscopy. Haploid and diploid strains bearing Cdc23-GFP and Apc9-GFP were viable (data not shown; see MATERIALS AND METHODS). Western analysis of Cdc23-GFP protein revealed a single band, indicating that Cdc23-GFP is a stable protein (data not shown). Since the GFP signal was severely diminished upon fixation, all studies were conducted using living budding yeast cells.

As shown in Figure 1, Cdc23-GFP was localized to the nucleus and one or two distinct foci within the nucleus (Figure 1A, I, II, and III). A punctate signal was also visible along the length of the mitotic spindle in anaphase (Figure 1A, III). No signal was detected in the "no GFP" control (data not shown). The localization patterns of Cdc23-GFP and Apc9-GFP were very similar (data not shown). However, the Apc9-GFP signal was much weaker than that of Cdc23-GFP and therefore more difficult to analyze. Therefore, Cdc23-GFP was used as the APC-GFP tag for the remainder of the experiments.

Since we initially observed that some cells displayed a diffuse nuclear signal, while others showed distinct nuclear foci within the background nuclear signal, we examined the localization pattern of Cdc23-GFP at different cell cycle stages (Figure 1A). To do this, we observed the Cdc23-GFP signal in live cells from asynchronous cultures, in addition to G_1 phase cells collected after centrifugal elutriation. Cells were grouped into three different categories (I–III) on the basis of their approximate cell cycle phase as judged by nuclear position and bud size (HARTWELL *et al.* 1974). Cdc23-GFP signal was concentrated in the nucleus at all cell cycle stages (Figure 1A). In addition to this homogeneous nuclear signal, we also observed punctate foci in the majority of cells at all cell cycle stages (Figure 1A). However, these foci did follow a particular localization pattern as the cell cycle progressed. In unbudded and small budded cells (Figure 1A, I), we detected a single nuclear spot. In cells with buds larger than one-third the size of the mother and in which the nucleus had migrated to the bud neck (Figure 1A, II), two spots could be seen. During early anaphase (Figure 1A, III) we observed a series of punctate spots arranged in a linear fashion along the anaphase spindle.

At each cell cycle stage, the percentage of cells with a visible nuclear spot signal varied, with the highest percentage showing nuclear spots in metaphase (II) and the lowest in anaphase (III) (Figure 1A). This Cdc23-GFP localization pattern was observed in the S288C and W303 strain backgrounds as well, and this localization pattern was not background dependent (data not shown). Therefore, with the exception of the appearance of Cdc23-GFP onto the anaphase spindle, Cdc23-GFP localization did not change dramatically during the cell cycle.

The Cdc28 cyclin-dependent kinase phosphorylates Cdc23p and at least two other APC/C subunits during mitosis (RUDNER and MURRAY 2000). We looked at the

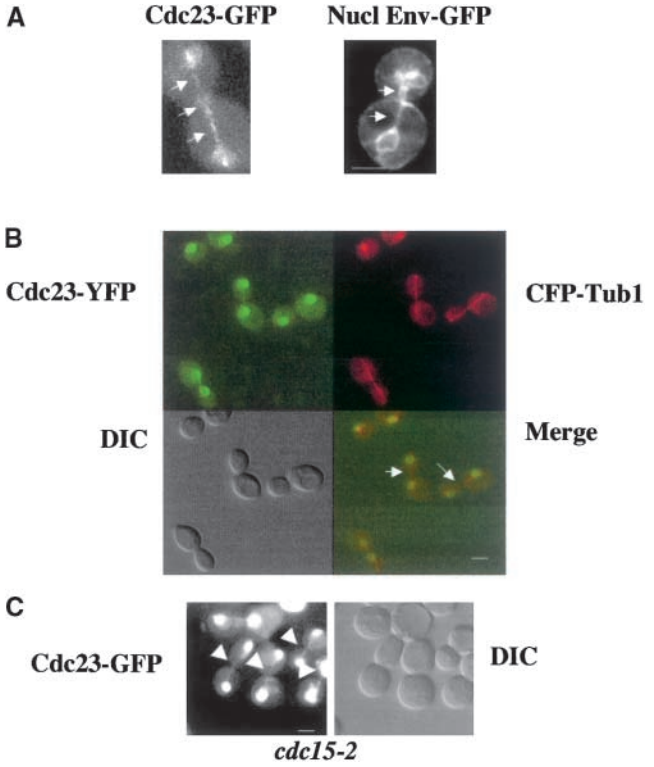

FIGURE 2.—The Cdc23-GFP and Cdc23-YFP expression pattern is consistent with an anaphase spindle signal. (A) The Cdc23-GFP signal (YSH1072) is compared to that of a cell expressing the ss-HDEL-GFP nuclear envelope marker (YSH1152). The anaphase spindle signal for Cdc23-GFP and the nuclear envelope signal for ss-GFP-HDEL are indicated by arrows. (B) CFP-Tub1 is colocalized with Cdc23-YFP (YSH1334). Arrows indicate colocalization along the presumptive anaphase spindle. (Contrast has been enhanced to bring out spindle signal.) (C) The Cdc23-GFP signal is observed in a temperature-sensitive mutant, *cdc15-2* (YSH1180), arrested in anaphase at 37°. Note the Cdc23-GFP spindle signal as indicated by arrowheads (image brightened to enhance visibility of spindle signal). Bar, 3 μm.

localization pattern of Cdc23p in a strain with all Cdc28p consensus sites mutated in the APC/C subunits Cdc23p, Cdc27p, and Cdc16p (RUDNER and MURRAY 2000). This particular mutant was impaired in the Cdc20-dependent phase of APC/C activity, a defect potentially linked to a change in APC/C localization. As indicated in Figure 1B, cdc23A-YFP expression was not dependent on Cdc28p-mediated phosphorylation, since its pattern did not change in an APC/C phosphorylation mutant ($n = 115$).

Colocalization of Cdc23-YFP with spindle and kinetochore markers, but not a spindle pole body marker: To narrow down the exact sites of Cdc23-GFP localization, we conducted colocalization studies using Cdc23-YFP and CFP-tagged spindle, spindle pole body, or kinetochore markers. In addition to colocalizing Cdc23-YFP with a spindle marker, we also analyzed the punctate linear Cdc23p pattern observed in anaphase cells in two other ways: comparing to a nuclear envelope GFP tag and using Cdc23-GFP in a *cdc15-2* mutant (Figure 2).

TABLE 3

Colocalization of CFP-Tub1 spindle signal and Cdc23-YFP

	Total (%)[a] ($n = 96$)
Colocalization	19 (90)
No colocalization	2 (10)
No Cdc23-YFP spindle-like signal (CFP-Tub1 signal present)	75

[a] Percentage of cells among those with visible Cdc23-YFP spindle signal.

To rule out the possibility that the nuclear envelope was responsible for the signal, we compared the Cdc23-GFP signal to that of a nuclear envelope marker, ss-GFP-HDEL (GAMMIE and ROSE 2002). The Cdc23-GFP signal pattern clearly differed from that of the nuclear envelope marker pattern in the width and uniformity of the signal (Figure 2A). Colocalization studies (Figure 2B; Table 3) performed using CFP-Tub1 and Cdc23-YFP confirmed an overlapping signal. CFP-Tub1 colocalized with 90% of Cdc23-YFP cells displaying a spindle-like signal (Table 3). However, many cells in anaphase that showed the typical CFP-Tub1 signal did not display a Cdc23-YFP spindle-like signal. This could have been due to the general weakness of the Cdc23-YFP relative to Cdc23-GFP and/or to the transient nature of the Cdc23-YFP spindle localization. Finally, a *cdc15-2* mutant strain containing Cdc23-GFP was used to enrich for late anaphase cells, a population expected to display a spindle localization of Cdc23-GFP (Figure 2C). This mutant arrested in late anaphase/telophase since the Cdc15p kinase was shown to be necessary for exit from mitosis (SCHWEITZER and PHILIPPSEN 1991; SURANA *et al.* 1993; JASPERSEN *et al.* 1998). As expected on the basis of earlier results, Cdc23-GFP was found along the anaphase spindle in the *cdc15-2* mutant at nonpermissive temperature (Figure 2C, arrowheads). Therefore, by colocalization analysis and a comparison to a nuclear envelope fluorescent marker, as well as by observing Cdc23-GFP using the *cdc15-2* mutant to enrich for anaphase cells, the linear Cdc23p anaphase signal did appear to colocalize with the anaphase spindle.

The cell-cycle-specific changes in the number of Cdc23-GFP nuclear spots suggested that the APC/C might be associated with spindle pole bodies, microtubule ends, or kinetochores in *S. cerevisiae* (KAHANA *et al.* 1995; GOSHIMA and YANAGIDA 2000; HE *et al.* 2001). To test these possibilities, we first conducted colocalization experiments with Spc42-CFP, a spindle pole body marker, and Cdc23-YFP (DONALDSON and KILMARTIN 1996). Cdc23-YFP did not colocalize with Spc42-CFP (Figure 3A). However, early and late in the cell cycle when kinetochores are close to the spindle pole bodies, separating the presumptive Cdc23-YFP spot from the spindle pole body spot was difficult (see Table 4). This ambiguity

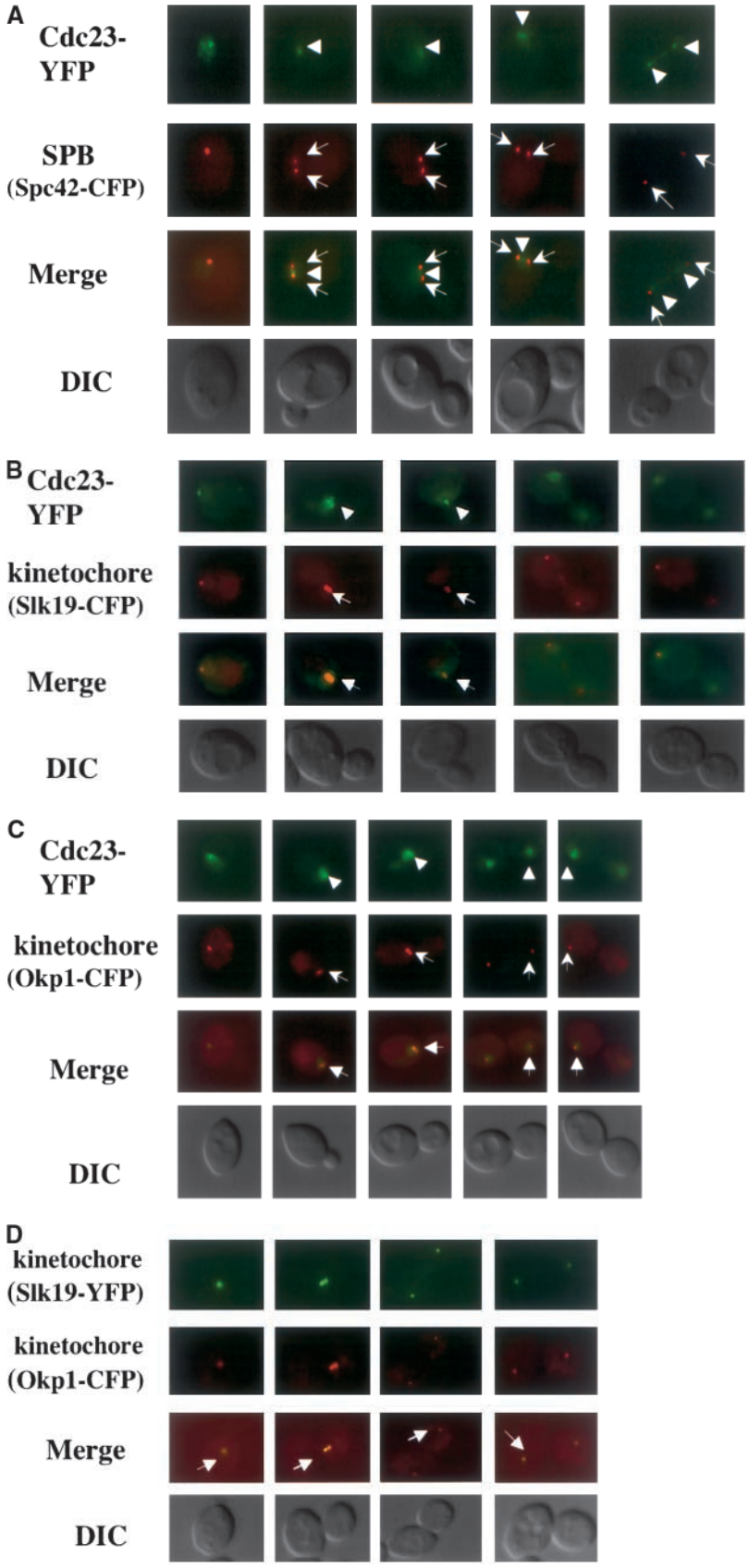

FIGURE 3.—Colocalization experiments using Cdc23-YFP with spindle pole body and kinetochore CFP-tagged markers. (A) Double-labeling experiments were conducted using Cdc23-YFP (top row) and Spc42-CFP (second row). The merge (third row) and the corresponding DIC (bottom row) are also shown. Cdc23p and Spc42p do not colocalize, with the differences most apparent in medium-budded cells. Arrowheads in top row, Cdc23-YFP spots; arrows in second row, Spc42-CFP spots. (B) Double-labeling experiments were done using Cdc23-YFP (top row) and Slk19-CFP (second row). The merge indicating colocalization (third row) and corresponding DIC image (bottom row) are also shown. Arrowheads in top row, Cdc23-YFP spots; arrows in second row, Slk19-CFP spots. (C) Double-labeling experiments were conducted using Cdc23-YFP (top row) and Okp1-CFP (second row). The merge indicating colocalization (third row) and corresponding DIC image (bottom row) are also shown. Arrowheads in top row, Cdc23-YFP spots; arrows in second row, Okp1-CFP spots. (D) Double-labeling experiments were conducted using Slk19-YFP (top row) and Okp1-CFP (second row). The merge (third row) and the corresponding DIC image (bottom row) are also shown. Arrows indicate colocalization spots. Slk19-YFP and Okp1-CFP colocalized 93% of the time (n = 43). For quantification of colocalization experiments shown in A–C, see Tables 4–6. SPB, spindle pole body.

TABLE 4

Colocalization of Spc42-CFP and Cdc23-YFP

	G_1 (%)[a] (n = 9)	S/Meta (%)[a] (n = 60)	Ana (%)[a] (n = 23)	Total (%)[a] (n = 92)
Colocalization	1 (20)	4 (10)	0 (0)	5 (10)
Partial colocalization	1 (20)	7 (18)	3 (60)	11 (22)
No colocalization	3 (60)	29 (72)	2 (40)	34 (68)
No Cdc23-YFP spots visible	4	20	18	42

[a] Percentage of cells among those with visible Cdc23-YFP spots.

was resolved in medium-budded metaphase cells, where Cdc23-YFP was clearly located between the two Spc42-CFP pole markers (see arrows in Figure 3A; Table 4). These results verified that the nuclear spots did not correspond to spindle pole bodies and led us to focus on whether these spots colocalized with a kinetochore marker. In fact, the Cdc23p localization pattern resembled the expression of known kinetochore components such as *SLK19* and *MTW1* (Zeng *et al.* 1999; Goshima and Yanagida 2000; Sullivan *et al.* 2001).

To determine whether or not Cdc23p colocalizes with a kinetochore marker, colocalization experiments were carried out using Slk19-CFP and Cdc23-YFP. As mentioned above, Slk19p is a kinetochore component that also moves to the spindle midzone in anaphase (Zeng *et al.* 1999; He *et al.* 2000, 2001; Pidoux and Allshire 2000; Sullivan *et al.* 2001). As seen in Figure 3B, Slk19-CFP colocalized with Cdc23-YFP, and this localization was clearest in medium-budded metaphase cells (Figure 3B, arrows; Table 5).

Additional colocalization studies were conducted using Cdc23-YFP and CFP-tagged Okp1p. Okp1p was biochemically and genetically determined to be a kinetochore component and a member of the Ctf19 kinetochore subcomplex (Ortiz *et al.* 1999; Cheeseman *et al.* 2002). However, its localization pattern had never been described prior to this study. Okp1p did interact with Cdc23p by two-hybrid analysis (P. G. Melloy and S. L. Holloway, unpublished results). Colocalization experiments of Okp1-CFP and Cdc23-YFP indicated that the two proteins colocalize throughout the cell cycle (Figure 3C, Table 6). Okp1-CFP also colocalized with the Slk19-YFP kinetochore marker (Figure 3D; n = 43).

Microtubules are required for the proper localization of Cdc23-GFP: We predicted that one possible mechanism of spindle checkpoint inhibition would be to mislocalize the APC/C. Conditions such as microtubule depolymerization and mutants to disrupt the spindle and kinetochore structure were used to test this hypothesis. First, cells expressing Cdc23-GFP or the GFP-tagged kinetochore component, Ndc10p, were treated with nocodazole. This drug depolymerized microtubules and resulted in spindle checkpoint activation (Shah and Cleveland 2000). Localization of Ndc10-GFP was expected to be unaffected by microtubule depolymerization (Goh and Kilmartin 1993). Both Cdc23-GFP and Ndc10-GFP localized to the kinetochore in medial nuclear division cells in the absence of nocodazole (Figure 4A, a and d; Figure 4B, g and i). However, in parallel cultures, the addition of nocodazole resulted in the delocalization of the punctate nuclear localization pattern of Cdc23-GFP but not that of Ndc10-GFP (Figure 4A, c and f; Figure 4B, h and j). For both Cdc23-GFP and Ndc10-GFP nocodazole-treated cells, visible spots often were slightly more diffuse or existed as a cluster of multiple spots (Figure 4A, b and e, Figure 4B, h and j).

Since nocodazole treatment also activated the spindle checkpoint in addition to depolymerizing microtubules, we wanted to see if the delocalization of the APC/C was due only to spindle checkpoint activation. We found that even in a spindle-checkpoint-defective *mad2*Δ mutant, Cdc23-GFP no longer retained its nuclear spot

TABLE 5

Colocalization of Slk19-CFP and Cdc23-YFP

	G_1 (%)[a] (n = 48)	S/Meta (%)[a] (n = 92)	Ana (%)[a] (n = 36)	Total (%)[a] (n = 176)
Colocalization	10 (67)	32 (70)	5 (63)	47 (68)
Partial colocalization	5 (33)	13 (28)	3 (37)	21 (30)
No colocalization	0 (0)	1 (2)	0 (0)	1 (2)
No Cdc23-YFP spots visible	33	46	28	107

[a] Percentage of cells among those with visible Cdc23-YFP spots.

TABLE 6

Colocalization of Okp1-CFP and Cdc23-YFP

	G_1 (%)[a] (n = 21)	S/Meta (%)[a] (n = 57)	Ana (%)[a] (n = 19)	Total (%)[a] (n = 97)
Colocalization	8 (80)	14 (42)	4 (36)	26 (48)
Partial colocalization	1 (10)	17 (52)	6 (54)	24 (44)
No colocalization	1 (10)	2 (6)	1 (10)	4 (8)
No Cdc23-YFP spots visible	11	24	8	43

[a] Percentage of cells among those with visible Cdc23-YFP spots.

signal (Figure 4C, f and h). On the basis of these experiments, it appeared that Cdc23-GFP localization was physically dependent on microtubules.

The Cdc23-GFP nuclear spot signal is delocalized in the *okp1-5* mutant: Since nocodazole affected microtubule structure as well as the spindle checkpoint, we wanted to test APC/C localization under conditions activating the checkpoint but not affecting microtubules. For this purpose we examined Cdc23-GFP localization in an *okp1-5* kinetochore mutant (Ortiz *et al.* 1999).

At nonpermissive temperature, the *okp1-5* mutant arrested at metaphase, but it was not previously known if this arrest was caused by spindle checkpoint activation. To make sure that the *okp1-5* mutant activated the checkpoint, we deleted the *MAD2* checkpoint gene in these cells to see if this relieved a checkpoint-activation-induced metaphase arrest. Deletion of *MAD2* has been previously shown to cause a failure of arrest (Bloecher and Tatchell 1999; Shah and Cleveland 2000; Gardner *et al.* 2001). The *okp1-5* mutant arrested in metaphase

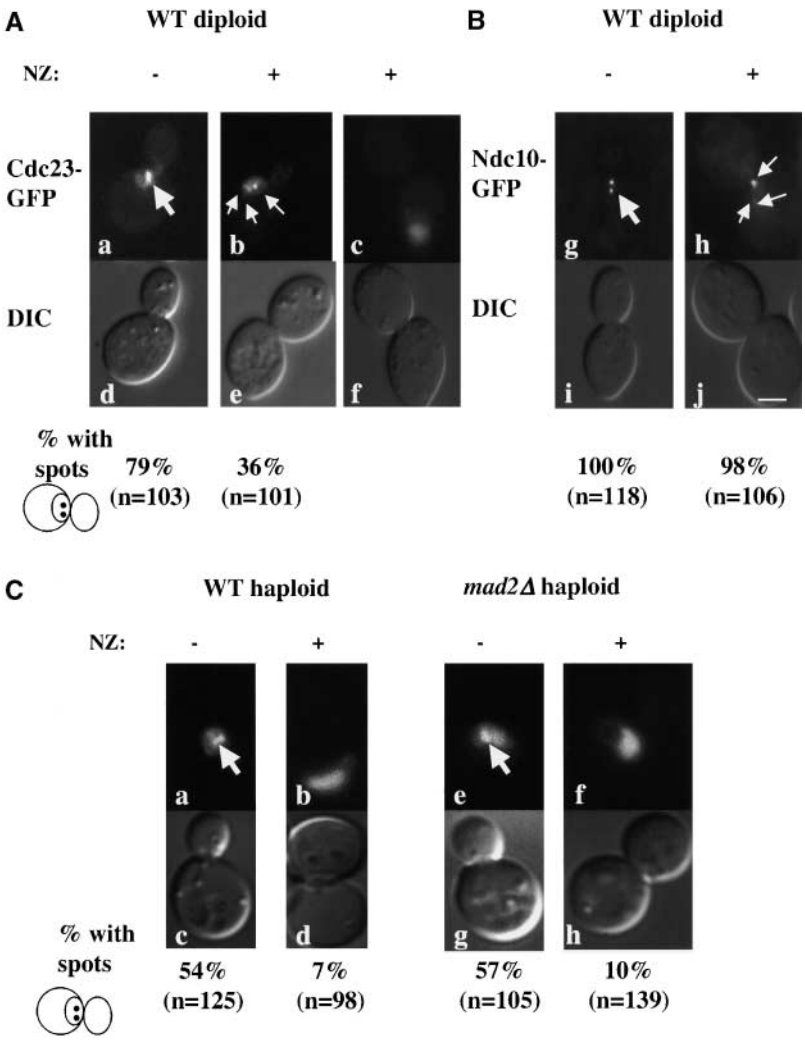

Figure 4.—The Cdc23-GFP nuclear spot localization requires microtubules. Nocodazole treatment of cells severely diminishes the Cdc23-GFP nuclear spot signal (YSH1190, YSH1072) but not the Ndc10-GFP (YSH1558) signal as indicated by the arrows in A for Cdc23-GFP and in B for Ndc10-GFP. Small arrows denote nocodazole-treated cells with multiple foci present. Listed below each image is the percentage of cells with one or more nuclear spots. Deletion of *MAD2* (YSH1753) does not prevent loss of the Cdc23-GFP nuclear spot signal (C). Bar, 3 μm.

at nonpermissive temperature as expected, while the *okp1-5mad2*Δ mutants failed to arrest and cycled into anaphase and G_1 (data not shown). From these results, it appeared that the *okp1-5* metaphase arrest resulted from spindle checkpoint activation, and this arrest was relieved in the absence of *MAD2*. The *okp1-5* mutant also had a synthetic phenotype with the *mad2*Δ spindle checkpoint mutant at 33° (data not shown). We tagged Cdc23p in the *okp1-5mad2*Δ mutant, but unfortunately the strain is too sickly to analyze the Cdc23-GFP kinetochore signal (data not shown).

After verifying spindle checkpoint activation in the *okp1-5* strain, we went on to observe the localization of Cdc23-GFP and the Slk19-GFP control protein in this mutant. At nonpermissive temperature, a threefold decrease in the Cdc23-GFP nuclear spot signal in metaphase cells was seen when compared to wild-type cells in metaphase (Figure 5A, b, d, f, and h). However, the signal of the Slk19-GFP protein was unchanged at nonpermissive temperature in this mutant (Figure 5B, j and l). Indirect immunofluorescence analysis revealed that although *okp1-5* spindle microtubules at 37° tended to be slightly longer than those at 23°, the spindles overall appeared normal and stable (see online supplemental Figure S1 at http://www.genetics.org/supplemental/). Therefore, disruption of the kinetochore and subsequent activation of the spindle checkpoint in an *okp1-5* mutant resulted in the displacement of the Cdc23-GFP nuclear spot signal.

The Cdc23-GFP nuclear spot localization at metaphase is dependent on Cdc20p: Since Cdc20p is a key regulator of the APC/C, it is important to show colocalization of these proteins at least during some point in the cell cycle. On the basis of our analysis of the endogenously tagged protein, Cdc20-GFP localization is consistent with a kinetochore localization from S phase until the end of mitosis (Figure 6, A and B).

We next looked at Cdc23-GFP localization in the *cdc20-1* mutant. Previous studies using this mutant indicated that it exhibits a metaphase arrest with a failure to degrade Pds1p, mitotic spindle defects, and a Bub2p-dependent spindle checkpoint arrest (Sethi *et al.* 1991; O'Toole *et al.* 1997; Visintin *et al.* 1997; Tavormina and Burke 1998). At nonpermissive temperature, this strain showed a ninefold decrease in cells with a Cdc23-GFP nuclear spot signal (Figure 6C, b and d). However, two other kinetochore proteins, Nuf2p and Okp1p, were not delocalized in this strain (Figure 6D, e and f and Figure 6E, g and h). These results suggested that either activation of the spindle checkpoint or defective microtubule function in a *cdc20-1* mutant led to a delocalization of the Cdc23-GFP nuclear spot signal. On the other hand, Cdc20p itself may have been needed to maintain the APC/C at the kinetochore, perhaps by degrading an unidentified target.

Cdc23-GFP localization is not changed in the checkpoint-sensor-defective *ndc10-1* mutant: Since we had previously shown that spindle checkpoint activation resulted in Cdc23-GFP delocalization from the kinetochore, we examined Cdc23-GFP expression in the *ndc10-1* mutant (Goh and Kilmartin 1993). Since *ndc10-1* mutants do not arrest in nocodazole, this mutant was known to have a defective spindle checkpoint sensor (Tavormina and Burke 1998; Gardner *et al.* 2001). In addition, binding of the yeast CBF3 kinetochore complex to centromeric DNA was severely compromised in this mutant (Sorger

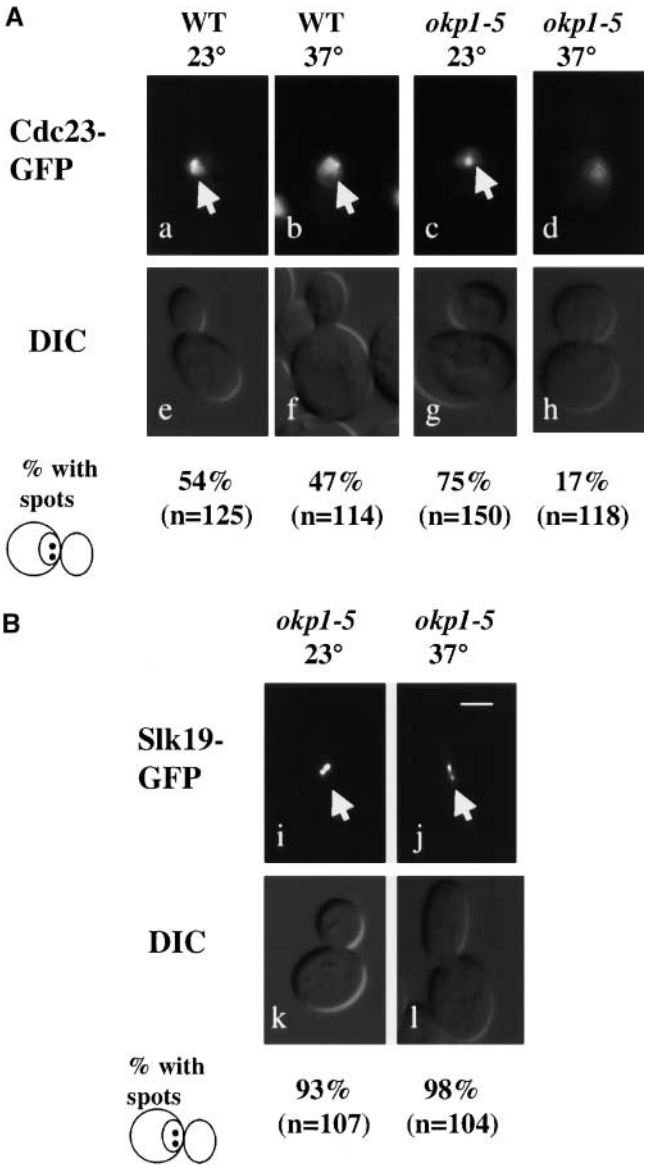

Figure 5.—The Cdc23-GFP nuclear spot signal is delocalized at metaphase in the *okp1-5* mutant. In A, Cdc23-GFP is delocalized in metaphase at nonpermissive temperature (37°) in the *okp1-5* mutant (YSH1565), but remains at the nuclear spots in the wild-type background-matched control (YSH1332; YSH1751) as indicated by arrows. As shown in B, the Slk19-GFP signal in the *okp1-5* mutant (YSH1582) remains unchanged even at nonpermissive temperature (arrows). The percentage of cells with one or two kinetochore spots is shown below each image for A and B. Bar, 3 μm.

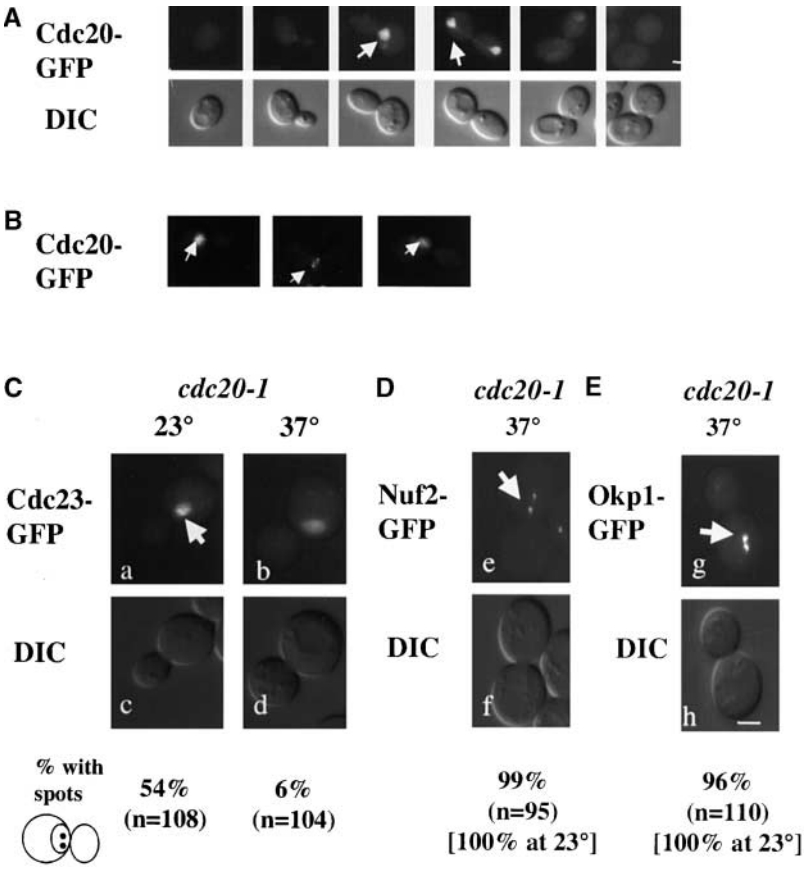

FIGURE 6.—Cdc20-GFP is expressed during mitosis in the nucleus, nuclear spots, and an anaphase spindle-like signal, and the Cdc23-GFP nuclear spot signal is disrupted in the *cdc20-1* mutant. Shown in A is the expression pattern of Cdc20-GFP (YSH1527) in representative images taken during different stages of the cell cycle ($n = 126$). Note the nuclear spot signal as indicated by arrows. In B, images of three metaphase cells with the nuclear spot signal are presented (spot signal indicated by arrows). (C) The decrease in Cdc23-GFP nuclear spot signal at nonpermissive temperature (37°) in a *cdc20-1* mutant (YSH1348; comparable background strain is YSH1332, Figure 5). Also note the micrographs in D and E, indicating that the Nuf2-GFP (YSH1588) and Okp1-GFP (YSH1587) signals do not change at 37°. Percentage of cells with one or two nuclear spots is indicated below each image. Bar, 3 μm.

et al. 1995). However, it was not known if this would affect Cdc23-GFP localization. In the case of at least five kinetochore-associated proteins, their kinetochore localization was lost in this mutant strain at nonpermissive temperature (He *et al.* 2001). We predicted that Cdc23-GFP would remain on the kinetochores if the checkpoint sensor were defective, but did not rule out that the perturbed kinetochore structure in the *ndc10-1* mutant may cause Cdc23-GFP to come off the kinetochore.

We localized Cdc23-GFP in the *ndc10-1* strain, along with two other kinetochore proteins, Nuf2-GFP and Okp1-GFP. Nuf2p was known to be delocalized from the kinetochore in an *ndc10-1* mutant, and Okp1p failed to bind centromeric DNA in a chromatin immunoprecipitation assay conducted using an *ndc10-1* mutant (Ortiz *et al.* 1999; He *et al.* 2001). Thus both Nuf2-GFP and Okp1-GFP were expected to come off the kinetochore in an *ndc10-1* mutant at nonpermissive temperature.

At permissive and nonpermissive temperature, a kinetochore signal for Cdc23-GFP was visible in the *ndc10-1* strain (Figure 7A, a–d). At permissive temperature, Nuf2-GFP and Okp1-GFP spots were visible (data not shown), but these spots disappeared at nonpermissive temperature (Figure 7A, e–h). So, despite perturbations in the kinetochore structure, Cdc23-GFP was not delocalized in the *ndc10-1* mutant. Since the *ndc10-1* mutation is a complex mutation known to affect the checkpoint response to damage such as nocodazole treatment, the APC/C may not be removed from the kinetochore if the sensor were damaged (Tavormina and Burke 1998).

Our localization analysis of Cdc23-GFP indicated that it remained in the nucleus and at nuclear spots colocalizing with the kinetochore throughout the cell cycle and was visible on the anaphase spindle during mitosis. Cdc23-GFP localization was affected by nocodazole treatment in the *okp1-5* mutant and in the *cdc20-1* mutant. In addition, failure of the kinetochore sensor in the *ndc10-1* mutant prevented delocalization of Cdc23-GFP from the kinetochore.

DISCUSSION

The APC/C subunit Cdc23p localizes to the nucleus, nuclear spots colocalizing with kinetochores, and the anaphase spindle: The observed Cdc23-GFP localization pattern was consistent with the role of the APC/C in ubiquitinating mitotic substrates and acting as a downstream component in the spindle checkpoint pathway. Recent studies using living cells revealed the presence of mammalian Cdc27 at the kinetochore, although this specific localization was not seen using fixed cells (Tugendreich *et al.* 1995; Topper *et al.* 2002). In addition, Drosophila GFP-Cdc27, human Apc10, and mouse Apc1 have been localized to either mitotic chromo-

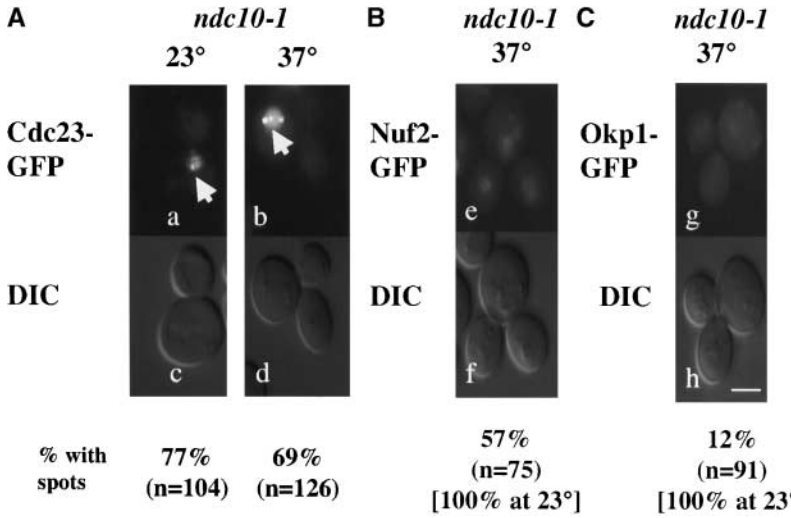

FIGURE 7.—The Cdc23-GFP nuclear spot signal is not displaced in an *ndc10-1* mutant. (A) The Cdc23-GFP (YSH1576) signal at permissive and nonpermissive temperature (37°) for the *ndc10-1* strain. Note that a spot signal (arrows) is visible at 37° for the Cdc23-GFP. However, as seen in B and C, no kinetochore spot signal is visible for Nuf2-GFP (YSH1592) and for the Okp1-GFP (YSH1590) signal at 37° in the *ndc10-1* strain. The percentage of cells with a nuclear spot signal is quantified below each image. Bar, 3 μm.

somes or kinetochores (JORGENSEN *et al.* 1998; KURASAWA and TODOKORO 1999; HUANG and RAFF 2002). Localization studies conducted using a variety of techniques, including observation of live cells, have increasingly shown that the APC/C localization pattern is highly conserved among various organisms.

In addition to conservation of APC/C localization, the spindle checkpoint components themselves have been localized to the kinetochore in several different organisms (LEW and BURKE 2003). In the case of some budding yeast checkpoint proteins, their kinetochore localization was dependent on checkpoint activation (IOUK *et al.* 2002; KERSCHER *et al.* 2003). Extensive analysis done in mammalian cells indicated that mammalian Mad2 associated with unattached, phosphorylated kinetochores (WATERS *et al.* 1999; HOWELL *et al.* 2000). At least two of these phosphorylated proteins at the mammalian kinetochore were APC/C subunits (DAUM *et al.* 2000). However, during mitosis itself in mammalian cells the dephosphorylated form of Cdc27 was found on the chromosomes (TOPPER *et al.* 2002). These experiments indicated that at least in mammalian cells, the phosphorylation state of the APC/C affected its regulation and localization. Although our studies indicated that the APC/C localized properly in an APC/C mutant lacking consensus phosphorylation sites for Cdc28p, inhibitory phosphorylation at other sites by other kinases may have an effect on APC/C activity during spindle checkpoint activation. Future studies will address checkpoint protein localization changes in relationship to APC/C localization.

Colocalization of Cdc23-YFP with kinetochore components is not surprising, since many mitotic regulators are found at the kinetochore. These proteins include cyclin B1, human Cdc20, and Mad2 (KALLIO *et al.* 1998, 2002; CLUTE and PINES 1999; HOWELL *et al.* 2000; KERSCHER *et al.* 2003). However, colocalization of Cdc23-YFP with the kinetochores throughout the cell cycle is surprising, because the APC/C has no known ubiquitination function outside of mitosis and G_1. Future studies may determine the significance of the APC/C nuclear spot localization during G_2 and S phases.

It is important to note that although Cdc23-YFP colocalizes with kinetochore components, we cannot rule out that the nuclear spot localization actually represents the microtubule ends. It is also possible that Cdc23p may lie at the interface between the kinetochore and the microtubule. Certain proteins known to associate with the outer kinetochore, such as the DASH complex member Ask1p, associate with the kinetochore in a microtubule-dependent manner, just like Cdc23p (LI *et al.* 2002).

Cdc23-GFP delocalization from the kinetochore or microtubule ends as a mechanism of the spindle checkpoint response: Our data suggest that the spindle checkpoint may halt the cell cycle not only by inhibiting APC/C activity, but also by changing its localization. The method of spindle checkpoint activation and the sensing of damage may be critical for APC/C displacement.

We activated the spindle checkpoint by several different mechanisms resulting in the delocalization of the Cdc23-GFP nuclear spot signal. These mechanisms included nocodazole treatment and the use of the *okp1-5* mutant. Because depolymerizing microtubules resulted in Cdc23-GFP localization regardless of spindle checkpoint status, several different methods of checkpoint activation were applied for this study. In each of our experimental conditions, the spindle or kinetochore damage itself did not appear to cause general protein displacement from the kinetochore, since control proteins such as Slk19-GFP and Ndc10-GFP consistently remained at the kinetochore during our experiments. In addition to these methods, we tested APC/C localization using the *cdc20-1* mutant. This mutant was known to activate the *BUB2*-dependent portion of the spindle checkpoint pathway, as well as to display defects in the organization of microtubules within the mitotic spindle (SETHI *et al.* 1991; O'TOOLE *et al.* 1997; TAVORMINA and BURKE 1998).

Delocalization of the Cdc23-GFP nuclear spot signal in this mutant may have been caused by a number of factors, including checkpoint activation, spindle structural changes, or a requirement of Cdc20p itself to maintain the APC/C at the kinetochore. In all cases of spindle checkpoint activation, delocalization of the nuclear spot Cdc23-GFP signal did not necessarily mean complete loss of APC/C activity, since the APC/C retained its general nuclear localization.

In addition to the previously mentioned methods of spindle checkpoint activation, we analyzed the localization of Cdc23-GFP and two kinetochore control proteins in response to *MPS1* overexpression (data not shown). Previous studies indicated that overexpression of this key regulatory kinase resulted in phosphorylation of Mad1p and spindle checkpoint activation even without spindle damage (Hardwick *et al.* 1996; Weiss and Winey 1996). The Cdc23-GFP nuclear spot signal was still visible upon *MPS1* overexpression, but in some cases seemed to move from a typical kinetochore nuclear position, yet remained as foci (data not shown). Therefore, these results were inconclusive. One possibility was that these foci represented movement of the APC/C along the microtubules to the spindle poles. Perhaps different methods of spindle checkpoint activation may have had slightly different effects on APC/C localization.

Recently, several articles showing the enrichment of certain checkpoint proteins at the kinetochore, such as Mad2p, upon spindle checkpoint activation have been published (Kerscher *et al.* 2003; Kitagawa *et al.* 2003; Gillett *et al.* 2004). However, little is known about how the amount of APC/C at the kinetochore or microtubule ends changes as these checkpoint proteins move to the kinetochore. Studies done in mammalian cells reveal that the APC/C and Mad2 may have overlapping Cdc20 binding sites (Zhang and Lees 2001). One might predict that as Mad2 moves to the kinetochore upon spindle checkpoint activation, the APC/C is eventually excluded from Cdc20p and is forced off the kinetochore or microtubule ends.

The role of the checkpoint sensor in APC/C localization: The checkpoint sensor appeared to be critical for APC/C regulation, since Cdc23-GFP was not displaced in the perturbed yet checkpoint-sensor-defective *ndc10-1* kinetochore mutant. However, it was unexpected that Cdc23-GFP remained at the kinetochore in an *ndc10-1* mutant, given the many examples of kinetochore proteins displaced in this strain (He *et al.* 2001).

It is surprising that Cdc23-GFP does not localize to the kinetochore/microtubule ends in an *okp1-5* mutant, but does in an *ndc10-1* mutant. This means that Cdc23-GFP must not be physically dependent on Okp1p to be bound to the kinetochore, since Okp1p itself does not bind the kinetochore in an *ndc10-1* mutant (Ortiz *et al.* 1999). The explanation for these data is most likely related to the activation of the spindle checkpoint sensor and to the inactivation of Cdc20p. Since Cdc20p may form a complex with Mad2p brought to the kinetochore upon spindle checkpoint activation, the APC/C may now come off the kinetochore in response to this activation. To address this issue, one could look at Cdc23-GFP localization in double mutants containing *mad2*Δ and another mutation activating the spindle checkpoint such as *cse4-1*, *ctf13-30*, or *dad1-1* (Biggins and Walczak 2003). In addition, Cdc23-GFP could be observed in other types of mutants with a failure of checkpoint activation, such as *spc24-1*, to see if this failure is directly responsible for the lack of Cdc23-GFP delocalization (Biggins and Walczak 2003).

It is also possible that if Cdc23-GFP is associated with the microtubule ends and not the kinetochore, or that if Cdc23-GFP moves onto the microtubule ends in an *ndc10-1* mutant, Cdc23-GFP could retain a nuclear spot localization at nonpermissive temperature. This is consistent with previous studies indicating that certain proteins associating with the kinetochore and the spindle can retain their spindle localization in an *ndc10-1* mutant (He *et al.* 2001; Janke *et al.* 2002; McAinsh *et al.* 2003).

APC/C localization in relation to its substrates and substrate-specificity factors: Cdc23-GFP appears to maintain its nuclear and nuclear spot localization during most of the cell cycle, but is visible on the spindle in anaphase. This spindle localization would position the APC/C for a "wave of proteolysis" as seen with cyclin B in Drosophila embryos (Huang and Raff 1999). It is possible that certain substrates are specifically shuttled to the spindle for ubiquitination, as in the case of mammalian cyclin B1 (Clute and Pines 1999). However, elimination of the spindle association of the motor protein Kip1p does not appear to prevent its ubiquitination (Gordon and Roof 2001). Although the association of an APC/C substrate with the spindle may not be essential for ubiquitination, the timing and specificity of ubiquitination may be altered if substrates are not properly targeted to the APC/C.

Cdc23-GFP is localized to many of the same locations as known APC/C substrates. However, Hsl1p, an APC/C substrate, is found at the bud neck and not at any of the observed APC/C locations (Burton and Solomon 2000, 2001). This particular substrate may need to be shuttled into the nucleus for degradation or ubiquitinated during nuclear migration through the neck. Kip1p and Cin8p are not degraded without a nuclear localization sequence, and certain nuclear transport proteins are necessary for Pds1p proteolysis (Baumer *et al.* 2000; Gordon and Roof 2001; Hildebrandt and Hoyt 2001). These observations support our localization data, suggesting that the primary site of APC/C activity is the nucleus. Future studies should address the significance of the specific localization of the APC/C within the nucleus, determining if certain substrates are stabilized when the APC/C is displaced.

In addition to the importance of APC/C localization

in determining APC/C activity, the localization of substrate-specificity factors such as Cdc20p and Hct1p may also be critical for APC/C regulation. Our studies indicate that Cdc20-GFP is localized to the nucleus and presumptive kinetochore from S phase to the end of mitosis. This localization pattern is very similar to studies conducted in mammalian cells, with a slight difference in the time period of kinetochore localization (Weinstein 1997; Kallio *et al.* 1998, 2002). Our findings also build on previous overexpression studies conducted in *S. cerevisiae* where Cdc20-GFP was described as nuclear during these same stages (Jacquenoud *et al.* 2002). Since the duration of Cdc20p localization to the nucleus and presumptive kinetochore is more restricted than that of the APC/C, the expression of Cdc20p in the nucleus and at the kinetochore may be a critical step in APC/C activation. Later stages of APC/C activity may also be tied closely to localization of a second specificity factor, Hct1p/Cdh1p. Although Hct1p is expressed throughout the cell cycle, the phosphorylated form remains in the cytoplasm during much of the cell cycle, with the dephosphorylated form present in the nucleus mainly during G_1 (Jacquenoud *et al.* 2002; Zhou *et al.* 2003). Clb2p proteolysis is impaired if Hct1p fails to enter the nucleus (Jacquenoud *et al.* 2002). Clearly localization of these specificity factors plays a critical role in APC/C activation and regulation.

Conclusions: In these studies, we have established that the *S. cerevisiae* APC/C subunit, Cdc23p, is localized to the nucleus, mitotic spindle, and either the kinetochore or the microtubule ends. These localization data are consistent with studies conducted in mammalian cells. Delocalization of the Cdc23-GFP nuclear spot signal upon spindle checkpoint activation also establishes a possible universal mechanism for APC/C inhibition.

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