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Mnd2 and Swm1 Are Core Subunits of the *Saccharomyces cerevisiae* Anaphase-promoting Complex*

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The anaphase-promoting complex (APC) is a multisubunit E3 ubiquitin ligase that regulates the metaphase-anaphase transition and exit from mitosis in eukaryotic cells. Eleven subunits have been previously identified in APC from budding yeast. We have identified two additional subunits, Mnd2 and Swm1, by mass spectrometry. Both Mnd2 and Swm1 were found specifically associated with a highly purified preparation of APC from haploid yeast whole cell extract. Moreover, the APC co-purified with epitope-tagged Mnd2 and Swm1. Both proteins were present in APC preparations from haploid cells arrested in G_1, S, and M phases and from meiotic diploid cells, indicating that they are constitutive components of the complex throughout the yeast cell cycle. Mnd2 interacted strongly with Cdc23, Apc5, and Apc1 when coexpressed in an *in vitro* transcription/translation reaction. Swm1 also interacted with Cdc23 and Apc5 in this system. Previous studies described meiotic defects for mutations in *MND2* and *SWM1*. Here, we show that *mnd2*Δ and *swm1*Δ haploid strains exhibit slow growth and accumulation of G_2/M cells comparable with that seen in *apc9*Δ or *apc10*Δ strains and consistent with an APC defect. Taken together, these results demonstrate that Swm1 and Mnd2 are functional components of the yeast APC.

The eukaryotic cell division cycle involves the replication of chromosomal DNA and its equal distribution to daughter cells in a highly regulated series of events. Failure to faithfully duplicate and segregate chromosomes can have dire consequences, such as the onset of cancer, in multicellular organisms. One of the essential regulatory components of chromosome segregation in eukaryotes is a large multisubunit enzyme termed the anaphase-promoting complex (APC),[1] or cyclosome (recently reviewed in Refs. 1 and 2). The APC is an E3 ubiquitin ligase responsible for initiating the metaphase to anaphase transition once chromosomes are attached and aligned at the metaphase plate and promoting mitotic exit once chromosome segregation is complete. The APC targets numerous substrate proteins involved in mitosis, meiosis, and other cellular processes for degradation by the proteasome by catalyzing their polyubiquitination (1) and is regulated by checkpoint signaling pathways that monitor DNA and chromosome integrity (3, 4).

Eleven constitutive core subunits of the APC have been identified in the budding yeast, *Saccharomyces cerevisiae* (5–7), and in vertebrates (8–11). Homologs of most of the subunits have been found in other model systems as well, including *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, and *Drosophila melanogaster* (reviewed in Ref. 2). Ten of the 11 known APC subunits of budding yeast have human homologs, with yeast Apc9 being the only exception. The extensive homology between APCs of organisms as diverse as humans and yeasts points to an ancient evolutionary origin and reflects the importance of the APC in controlling some of the most fundamental cell cycle events in eukaryotes.

The presence of so many subunits makes the APC an unusual E3 enzyme in terms of its size and complexity. The actual catalytic reaction involving transfer of ubiquitin from an E2 enzyme to a substrate protein is intrinsic to a single small RING finger subunit, Apc11 (11, 12). Another subunit, Apc2, containing a highly conserved cullin domain present in other E3 ubiquitin ligases interacts with Apc11 (13) and is also believed to be important for catalyzing ubiquitin transfer (14). The specific functions of the remaining subunits are almost entirely unknown. Candidate roles for these components include substrate recruitment and specificity, cellular localization, or interaction with and response to regulatory proteins such as cyclin-dependent kinases and spindle assembly checkpoint proteins (2). Some subunits may function in a purely structural manner by forming a scaffold that allows proper complex assembly.

In our efforts to purify the APC from budding yeast extracts, we consistently observed two previously unidentified proteins co-purifying under high salt conditions with the core complex. Here, we provide evidence that these two proteins, Mnd2 and Swm1, are, in fact, constitutive and functional components of the core APC, bringing the total number of identified subunits in budding yeast to 13. We believe that Swm1 is identical to Apc13, a small protein observed previously in APC preparations that was never identified (7). We discuss the significance of these identifications, the previously described meiotic defects associated with *MND2* and *SWM1* mutations (15, 16), and the mitotic phenotypes we have observed in *mnd2*Δ and *swm1*Δ strains for understanding aspects of APC function in all organisms.

EXPERIMENTAL PROCEDURES

Yeast Methods and Strain Construction—Yeast strains expressing Cdc27, Swm1, and Mnd2 (Table I) containing carboxyl-terminal 3× FLAG epitopes were constructed by integration of PCR products amplified from the template p3FLAG-KanMX (gift from Dr. Toshio Tsukiyama; Fred Hutchinson Cancer Center) at the desired location as described (17). Integrants were selected on YPD agar containing 500 μg/ml G418, and correct integration of the cassette was confirmed by

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[1] The abbreviations used are: APC, anaphase-promoting complex; RTS, rapid translation system; MS, mass spectrometry; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase.

TABLE I
S. cerevisiae strains used in this study

Strain	Relevant genotype	Source
W1588-4c[a]	*MAT*a *ade2–1 can1–100 His3–11,15 leu2–3, 112 trp1–1 ura3–1*	R. Rothstein
YKA151	*CDC27–3FLAG*:KanMX4	This study
YKA152	*MND2–3FLAG*:KanMX4	This study
YKA153	*SWM1–3FLAG*:KanMX4	This study
YKA155	*CDC27–3FLAG*:KanMX4 *bar1*Δ::*URA3*	This study
BY4741	*MAT*a*His3D1 leu2D0 met15D0 ura3D0*	SGDP[b]
2713[c]	*apc9*Δ::KanMX4	SGDP[b]
4607[c]	*apc10*Δ::KanMX4	SGDP[b]
5960[c]	*mnd2*Δ::KanMX4	SGDP[b]
3619[c]	*swm1*Δ::KanMX4	SGDP[b]
YPH499	*MAT*a *ura3–52 lys2–801 ade2–101 trp1-Δ63 his3-Δ200 leu2-Δ1*	Stratagene
YKA180	*MAT*a/α *CDC27–3FLAG*:KanMX4	This study

[a] W1588-4c is a derivative of W303 in which the weak *rad5* mutation has been repaired (25)
[b] SGDP, *Saccharomyces* Genome Deletion Project.
[c] These strains were all derived from BY4741, and the values correspond to the *Saccharomyces* Genome Deletion Project record number.

PCR and DNA sequencing. Deletion of the *BAR1* gene from W1588-4c was achieved by integration of a *URA3* cassette amplified by PCR from pRS406 at the *BAR1* locus. Replacement of *BAR1* with *URA3* was confirmed by PCR. Diploid strain YKA180 was created by transformation of YKA151 with YCp50::HO expressing the wild-type HO endonuclease, selecting for transformants on medium lacking uracil and then counterselecting for loss of YCp50::HO on medium containing 5-fluoroorotic acid. The diploid strain was confirmed by its ability to sporulate. Strains from which the *APC9*, *APC10*, *SWM1*, or *MND2* genes had been deleted (Table I) as well as their parent strain, BY4741, were from the *Saccharomyces* Genome Deletion Project, available through ResGen. The presence of the correct deletion was confirmed in each of these strains by PCR using primers flanking the appropriate open reading frame.

Cell cycle arrests were performed in midlog phase cultures of strain YKA155 as follows. For G_1 arrest, α-factor peptide (University of North Carolina peptide synthesis facility) was added from a 5 mg/ml stock in ethanol to a final concentration of 50 μg/liter. For S arrest, hydroxyurea (Sigma) powder was added directly to cultures at a final concentration of 10 mg/ml. For M arrest, nocodazole (Sigma) was added from a 1.5 mg/ml stock in dimethyl sulfoxide to a final concentration of 15 μg/ml. Cell cycle arrests were monitored by phase-contrast microscopy until >90% of the cells had achieved the desired morphology (unbudded for G_1 and large budded for S and M). Samples were removed from the cultures and analyzed by flow cytometry on a FACScan flow cytometer (Becton Dickinson) to confirm arrest at the desired stage. Diploid cells were induced to enter a synchronous meiosis exactly as described previously (18). Cells were harvested midway through meiosis based on the meiotic progression of yeast strain W303 described recently (19). Induction of sporulation was confirmed by monitoring spore formation by microscopy.

RTS Expression Constructs—Expression plasmid pIVEX-2.3d and all Rapid Translation System (RTS) reagents were from Roche Applied Science. To construct pIVEX-FLAG, oligonucleotides 5′-CATGGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGATGACAAGGGCGGAGGAGC-3′ and 5′-GGCCGCTCCTCCGCCCTTGTCATCGTCATCCTTGTAATCGA TGTCATGATCTTTATAATCACCGTCATGGTCTTTGTAGTC-3′ were annealed in 10 mM Tris-HCl, pH 8.5, by heating to 95 °C and slowly cooling to 30 °C. The annealed product was ligated into the *Nco*I and *Not*I restriction enzyme sites of pIVEX-2.3d.

Yeast open reading frames for the 11 previously identified APC subunits as well as *SWM1* and *MND2* were amplified by PCR using Platinum *Pfx* DNA polymerase (Invitrogen) and yeast genomic DNA from strain YPH499 (Stratagene) as the template. Oligonucleotide primers contained restriction enzyme sites to facilitate ligation into either pIVEX-FLAG or pIVEX-2.3d. The resulting pIVEX-FLAG and pIVEX-2.3d constructs allow expression of APC subunits containing an N-terminal 3× FLAG epitope or a C-terminal His_6 sequence, respectively, using RTS *in vitro* transcription and translation reactions. The 5′ and 3′ junctions of all clones and the identities of the cloned open reading frames were confirmed by DNA sequencing.

Purification of APC—Approximately 10^{11} cells from late log phase cultures were washed with H_2O and resuspended in 200 ml of cold (4 °C) APC buffer (25 mM HEPES-NaOH, pH 7.5, 400 mM NaCl, 10% glycerol, 0.1% Triton X-100, 0.5 mM dithiothreitol, 25 mM NaF, 25 mM β-glycerophosphate, and 1 mM activated sodium orthovanadate) containing freshly added complete protease inhibitor tablets (Roche Applied Science) and 0.5 mM phenylmethylsulfonyl fluoride. All subsequent steps were performed at 4 °C or on ice. Cells were disrupted six times for 1.5 min with 0.5-mm glass beads in a bead beater (Biospec Products), allowing 5 min between pulses for cooling. Extract (~200 ml) was precleared by centrifugation for 30 min at 35,000 × *g* and cleared a second time for 1 h at 92,000 × *g*. The soluble extract (5–15 mg/ml protein) was incubated with 100 μl of pre-equilibrated EZview anti-FLAG M2 antibody-coupled agarose resin (Sigma) for 2 h. Beads were collected by centrifugation, washed four times for 10 min with 25 ml of APC buffer, transferred to a microcentrifuge tube, and washed an additional three times with 1 ml of APC buffer. APC was eluted by two sequential 30-min incubations at 30 °C with 200 μl of APC buffer containing 500 μg/ml 3× FLAG peptide (Sigma). Elutions were pooled, and APC was precipitated with 6 volumes of acetone.

For the peptide block control, an extract from YKA151 was split into two equal volumes. From one half, APC was purified as described above. From the other half, APC was purified using an identical volume of anti-FLAG affinity resin that had been blocked by incubation with 1 ml of 500 μg/ml 3× FLAG peptide in APC buffer for 1 h before the addition to the extract. Otherwise, the two preparations were performed identically.

Identification of SDS-PAGE Gel Bands by Mass Spectrometry (MS)—Proteins eluted from the anti-FLAG affinity resin were separated by SDS-PAGE on 4–12% gradient NuPAGE gels (Invitrogen) and stained with Coomassie Brilliant Blue R-250 (Bio-Rad). Individual gel bands were carefully excised with a razor and subjected to trypsin proteolysis using a ProGest automated digester (Genomic Solutions). Extracted tryptic peptides were analyzed on a Reflex III matrix-assisted laser desorption ionization time-of-flight mass spectrometer (Bruker Daltonics). Data were internally calibrated with trypsin autoproteolysis peaks and submitted to the MASCOT database search engine (Matrix Science) for protein identification by peptide mass fingerprinting. All identifications in this study represent statistically significant matches from the database of *S. cerevisiae* proteins. When a statistically significant match was not obtained by peptide mass fingerprinting, individual peptides from the spectrum were subjected to nanoelectrospray tandem MS on a QStar mass spectrometer (Applied Biosystems) to confirm the identity of the protein.

APC Subunit Interaction Assay—RTS *in vitro* transcription and translation reactions were performed according to the supplied instructions. Two APC subunits, one containing the 3× FLAG epitope and the other containing a His_6 tag were coexpressed for 5 h at 30 °C in 50-μl reactions containing ~0.3 μg of each expression plasmid. Insoluble protein was removed by centrifugation, and 40 μl of the soluble material was diluted to 500 μl with RTS buffer (25 mM HEPES-NaOH, pH 7.5, 150 mM sodium acetate, 10% glycerol, 0.1% Nonidet P-40, and 0.5 mM dithiothreitol). The sample was cleared a second time by centrifugation, and the supernatant was incubated with 10 μl of pre-equilibrated anti-FLAG affinity resin for 1 h at 4 °C. The resin was washed three times with 1 ml of RTS buffer, and specifically bound protein was eluted with 40 μl of 500 μg/ml 3× FLAG peptide in RTS buffer overnight at 4 °C. Eluted proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes, and the presence of the His_6-tagged protein was evaluated by Western blot with anti-His_6 polyclonal antibody (Covance). Membranes were stripped and reprobed with anti-FLAG M2 monoclonal antibody (Sigma) to ensure that the immunoaffinity purifications were successful. Expression of both proteins in the

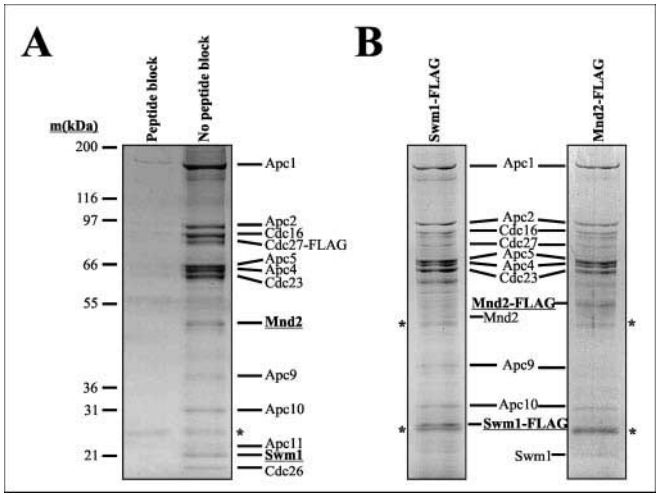

FIG. 1. **Mnd2 and Swm1 copurify with yeast APC.** Immunoaffinity purifications of APC from yeast whole cell extracts were separated by SDS-PAGE as described under "Experimental Procedures" and stained with Coomassie Blue. Each *lane* represents the material obtained from $\sim 10^{11}$ cells. Individual bands were excised and digested with trypsin, and the proteins were identified by mass spectrometry. All labeled proteins represent statistically significant scores obtained by the MASCOT search engine. *A*, APC was prepared from strain YKA151, which produces 3× FLAG epitope-tagged Cdc27. The samples in both lanes were treated identically except that the anti-FLAG beads in the first sample were preblocked with 3× FLAG peptide before incubation with the cell extract. *B*, APC was purified from strain YKA152, which produces 3× FLAG epitope-tagged Mnd2, and strain YKA153, which produces 3× FLAG epitope-tagged Swm1. Bands labeled with an *asterisk* in each preparation were identified as mouse IgG. Most of the visible but faint unlabeled bands on these gels were identified as proteolytic fragments of APC subunits.

reactions was also confirmed by Western blot using 5 μl of the original reaction.

Growth Curves and Flow Cytometry—To measure relative growth rates of strains harboring deletions of *APC9*, *APC10*, *SWM1*, or *MND2* as well as the isogenic wild-type strain, three individual colonies of each strain were grown overnight in 5 ml of YPD at 30 °C. Cultures were diluted 20-fold in YPD and allowed to grow at 30 °C for 3 h. For growth curves at 37 °C, cultures were transferred to 37 °C and incubated for 1 h before beginning measurements. Cultures were diluted to identical starting densities of 3×10^5 cells/ml for 30 °C experiments or 5×10^5 cells/ml for 37 °C experiments, and at the indicated time points OD_{660} measurements were taken. OD_{660} values were converted to cell density for graphical display of growth curves. Samples from each culture were removed at OD_{660} ~0.5 and prepared for analysis by flow cytometry.

For flow cytometry, cells from 0.5 ml of culture were washed with 1 ml of H_2O and fixed overnight in 1 ml of 70% ethanol at 4 °C. Cells were rinsed twice with 1 ml of 50 mM Tris-HCl, pH 8.0, and incubated for 2–3 h at 50 °C with 2 μg/ml RNase A and 1 mg/ml proteinase K in 50 mM Tris-HCl. After washing with 1 ml of FC buffer (200 mM Tris-HCl, pH 8.0, 200 mM NaCl, 78 mM $MgCl_2$), cells were resuspended in 500 μl of FC buffer containing 5 μM Sytox Green (Molecular Probes, Inc., Eugene, OR). DNA content was measured on a FACScan instrument. Percentages of G_1, S, and G_2/M cells were calculated using ModFit LT software (Verity Software House, Inc.).

RESULTS

Identification of Two Unknown Proteins Co-purifying with Yeast APC—We constructed a yeast strain, YKA151, which produces the Cdc27 protein with a carboxyl-terminal 3× FLAG epitope tag from its natural chromosomal locus for immunoaffinity purification of the anaphase-promoting complex. In our preparations of APC from YKA151, we identified all 11 known APC subunits by peptide mass fingerprinting or tandem MS as well as two other bands that had not been previously described as APC subunits (Fig. 1*A*, *second lane*). We also observed these two proteins associated with APC in preparations from a yeast strain containing a 6-Myc epitope tag on the Cdc16 APC subunit (data not shown). It should be noted that the conditions used for the affinity purification of APC include a high salt concentration (425 mM Na^+) at all steps, demonstrating the high salt stability of the APC. To determine whether these proteins were specifically associated with our purified APC as opposed to nonspecifically associated with the anti-FLAG affinity resin, we performed a control purification in which the anti-FLAG affinity beads were preblocked with the antigenic 3× FLAG peptide (Fig. 1*A*, *first lane*). Both proteins were effectively competed away by the blocking peptide, suggesting that their presence was due to direct interaction with the APC. The two proteins, Mnd2 and Swm1, have both been implicated in meiosis, but at different stages (15, 16). Little else is known about them.

Known APC Subunits Co-purify with FLAG Epitope-tagged Mnd2 and Swm1—To provide more convincing evidence that Mnd2 and Swm1 are true subunits of the APC, we constructed yeast strains producing 3× FLAG-tagged versions of Mnd2 and Swm1 (YKA152 and YKA153, respectively). We subjected whole cell extracts from these strains to the same stringent immunoaffinity purification protocol used with YKA151. Proteins in these preparations were identified from Coomassie-stained polyacrylamide gels by peptide mass fingerprinting. In both cases, we identified 9 of the 11 known APC subunits co-purifying with the epitope-tagged protein (Fig. 1*B*). Apc11 and Cdc26 were not identified; however, these two small subunits generally required tandem MS for identification due to the low number of tryptic peptides generated. Regardless, these results conclusively demonstrate that Mnd2 and Swm1 are stably associated with the core APC in haploid yeast cells.

Mnd2 and Swm1 Are Constitutive Components of the APC during the Cell Cycle—Although its activity fluctuates, the APC is a stable complex that is present throughout the cell cycle (8, 10). To determine whether Mnd2 and Swm1 are also associated with the APC during different cell cycle stages, APC was immunoaffinity-purified from cell cultures arrested in G_1 phase with α-mating factor, in S phase with hydroxyurea, and in M phase with nocodazole (Fig. 2, *A–C*). Mnd2 and Swm1 were identified by mass spectrometry and were present in approximately equal abundance at all three cell cycle stages with respect to the abundance of the other APC subunits, judging from the intensity of Coomassie-stained gel bands. These results suggest that, like the other 11 components, Mnd2 and Swm1 are constitutively associated with the APC and can therefore be considered core subunits.

Given the fact that lack of *MND2* and *SWM1* has previously been associated with severe meiotic defects (15, 16), we created a diploid strain expressing *CDC27* with three copies of the FLAG epitope to determine whether Mnd2 and Swm1 are components of the APC during meiosis. The diploid cells were induced to sporulate in a synchronous manner according to a previously described method (18), and cells were harvested midway through meiosis (19). APC was purified from the meiotic cells using our standard purification procedure (Fig. 2*D*), and the subunits were identified by mass spectrometry. Both Mnd2 and Swm1 were present along with the other known APC subunits. Although we cannot rule out the possibility that Mnd2 or Swm1 dissociates transiently from the APC at a specific stage of meiosis to carry out an APC-independent function, our results support the conclusion that they remain associated with the APC during the sporulation program.

Identification of Subunit-Subunit Contacts Involving Mnd2 and Swm1—We established an interaction assay based on co-expression of two APC subunits, each with a different affinity tag, in an *E. coli*-derived *in vitro* transcription and translation system (see "Experimental Procedures"). In this assay, one

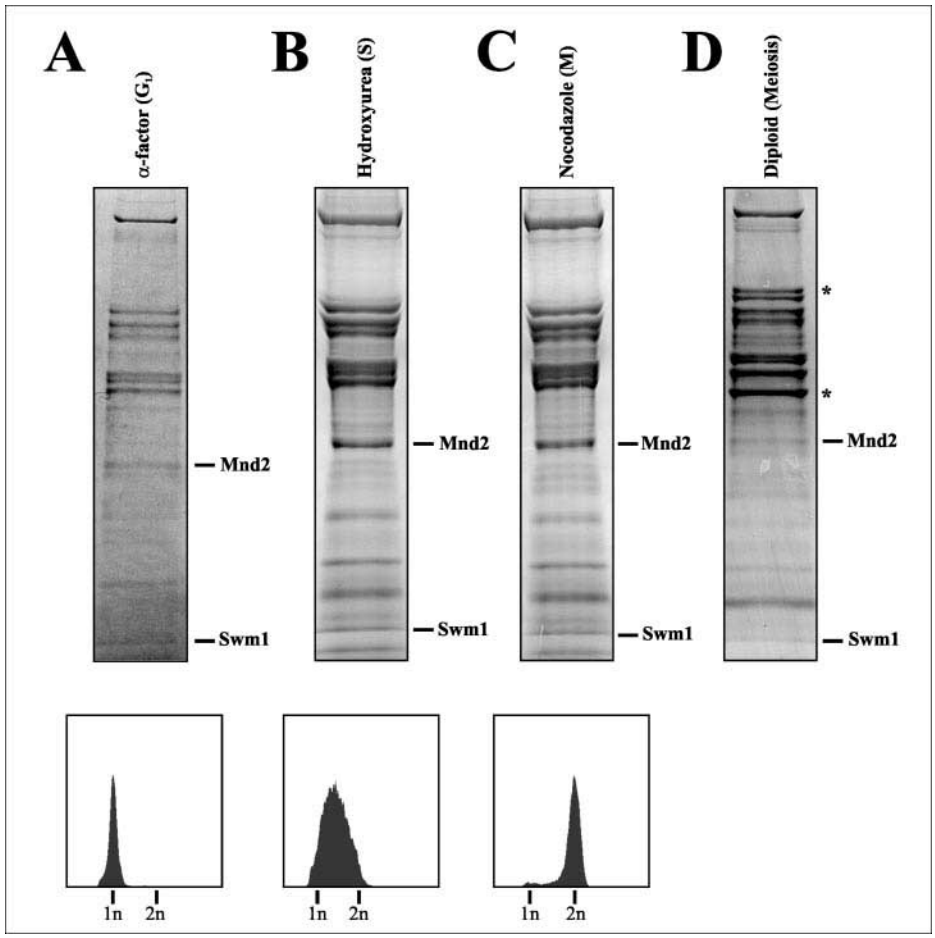

FIG. 2. **Mnd2 and Swm1 are constitutive components of yeast APC.** APC was immunoaffinity-purified as described under "Experimental Procedures" from haploid yeast cell cultures arrested in G_1 phase with α-factor peptide (*A*), S phase with hydroxyurea (*B*), or M phase with nocodazole (*C*) or from diploid cells induced to undergo meiosis (*D*). The purified APC in each case was separated by SDS-PAGE, and proteins were visualized by staining with Coomassie Blue. The presence of Mnd2 and Swm1 in each of the four preparations was confirmed by mass spectrometry. A small sample of cells from each of the haploid cultures was analyzed by flow cytometry to confirm arrest at the desired cell cycle stage. Histograms of the DNA content in these samples are displayed *below* the corresponding preparation. *1n*, one copy of the genome; *2n*, two copies of the genome. The *two asterisks* in the diploid preparation (*D*) indicate prominent bands that were not apparent in the haploid preparations. The larger of the two proteins was identified as 6-phosphofructo-2-kinase, which happens to contain a consensus FLAG epitope within its amino acid sequence. This protein was also observed in our haploid preparations at lower levels. The smaller of the *asterisk-labeled bands* was identified as Ach1p, which is involved in acetate metabolism and is probably a nonspecific contaminant that appears because the sporulating cells use acetate as a carbon source.

APC subunit is expressed as a fusion with the 3× FLAG epitope, and the second subunit is expressed as a fusion with a His_6 tag. Following the reaction, the subunit containing the FLAG epitope is purified using anti-FLAG antibody-coupled beads, and the presence or absence of the second subunit is monitored by Western blot with anti-His_6 antibody. A positive signal in the anti-His_6 Western blot is indicative of a physical interaction between the two subunits that results in their copurification.

We screened Mnd2 and Swm1 for interactions with as many of the other APC subunits as possible, including homodimeric interactions and interactions with each other. First, we tested Mnd2-His_6 by coexpression with a series of FLAG-tagged APC subunits. In this assay, Mnd2-His_6 interacted with Apc1-FLAG, Apc5-FLAG, and Cdc23-FLAG (Fig. 3*A*). The data also suggested a weak interaction with Apc2-FLAG. However, the signal was substantially weaker than the other three interactions, and in light of the fact that Apc2-FLAG expression was greater than most of the other subunits (Fig. 3*A* and data not shown), we cannot definitively conclude that this result represents a *bona fide* interaction.

Since we were unable to make a construct capable of expressing Swm1-His_6 in the RTS system, we screened Swm1-FLAG against a collection of His_6-tagged APC subunits to identify its interaction partners. In this experiment, Swm1-FLAG interacted with Apc10-His_6, Cdc23-His_6, and Apc5-His_6 (Fig. 3*B*). Control reactions to evaluate the specificity of these apparent interactions (Fig. 3*C*) revealed that the Apc10-His_6 signal in the elution was not dependent on coexpression of Swm1-FLAG and probably reflected nonspecific association with the antibody resin. On the other hand, the Cdc23-His_6 and Apc5-His_6 signals were dependent on coexpression of Swm1-FLAG, and we can conclude that Swm1 physically interacts with Cdc23 and Apc5.

We were unable to evaluate potential interactions with Apc11, because Apc11 expressed in this system consistently gave artifactual results (not shown). Also, we were unable to evaluate the potential interactions Mnd2-Cdc27, Swm1-Swm1, or Swm1-Apc1 because we could not generate clones that would express Cdc27-FLAG, Swm1-His_6, or Apc1-His_6 in the RTS system. Nonetheless, we were able to identify at least a portion of the subunit contacts that are probably responsible for the stable association of Swm1 and Mnd2 with the APC *in vivo*.

Deletion of MND2 or SWM1 Results in Slow Growth Associated with Accumulation of G_2/M Cells—Defects in APC function generally result in cell cycle arrest in metaphase or a delay in progression of the cell cycle through mitosis. We compared the growth rates of *swm1*Δ and *mnd2*Δ haploid strains to strains containing deletions of two other nonessential APC genes, *apc9*Δ and *apc10*Δ, as well as the isogenic wild-type strain. At 30 °C, *apc9*Δ, *mnd2*Δ, and *swm1*Δ all exhibited modest but statistically significant slow growth phenotypes, whereas the growth defect of *apc10*Δ was much more acute (Fig. 4*A*). At 37 °C, the slow growth phenotypes of *apc9*Δ, and *mnd2*Δ were slightly more severe compared with the parental strain. However, the severity of the *swm1*Δ growth defect was greatly increased at 37 °C, nearly to the level of *apc10*Δ (Fig. 4*B*).

In an effort to pinpoint the cause of the slow growth phenotype of *swm1*Δ and *mnd2*Δ, cells from each of the five strains were taken during the growth curve experiment at 37 °C, and their DNA content was analyzed by flow cytometry. The *apc9*Δ strain exhibited a slight but noticeable and statistically significant accumulation of G_2/M cells compared with the wild-type

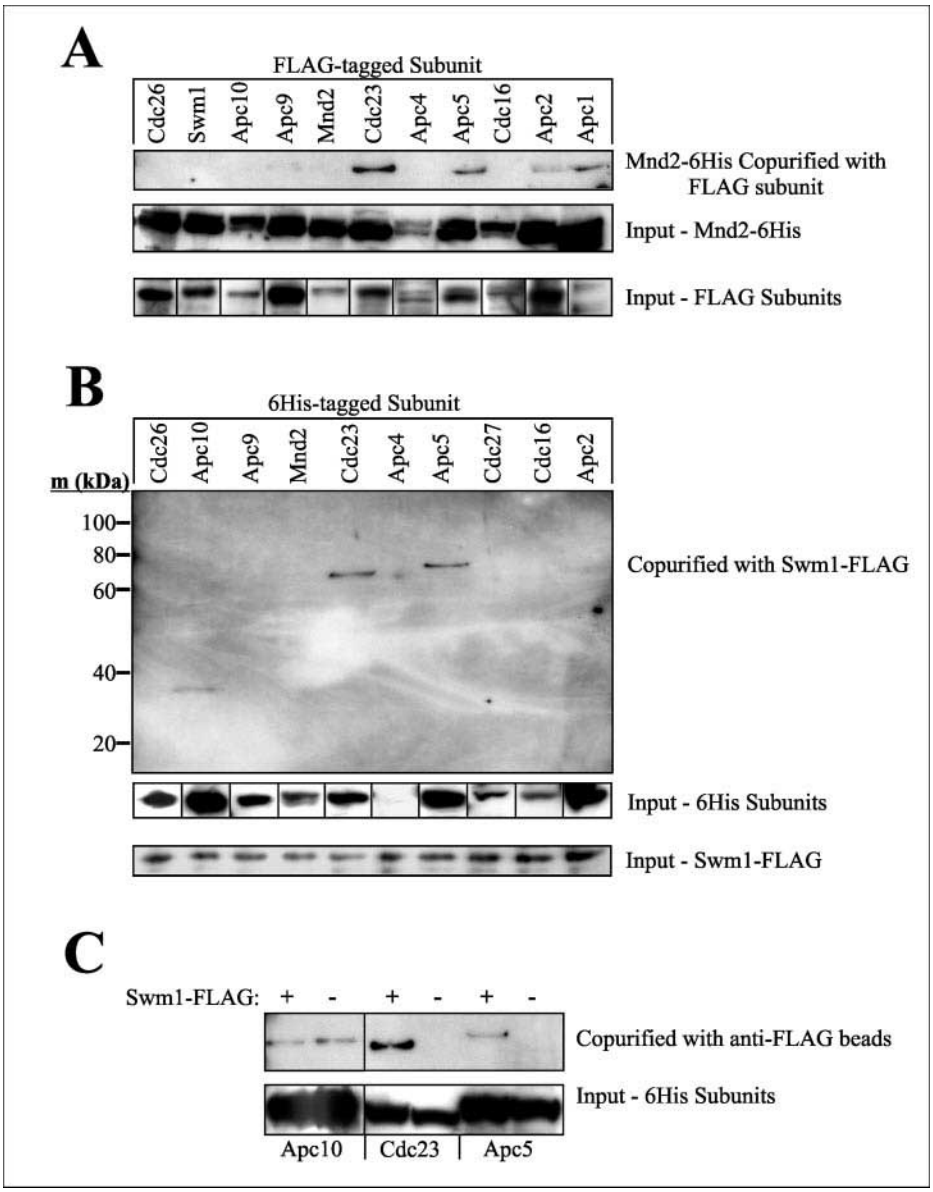

FIG. 3. **Subunit-subunit interactions involving Mnd2 and Swm1.** *A*, Mnd2-His_6 protein was coexpressed with each of the indicated 3× FLAG epitope-tagged APC subunits in an *E. coli*-based *in vitro* transcription and translation reaction as described under "Experimental Procedures." Expression of each protein was confirmed by Western blot using either anti-His_6 polyclonal or anti-FLAG monoclonal antibodies (*Input*). The Mnd2-His_6 band in the *Input* blot appears as a doublet, but the upper band is a cross-reacting protein from the *E. coli* lysate. FLAG-tagged subunits were purified using anti-FLAG affinity resin and specifically eluted with 3× FLAG peptide. Co-purification of Mnd2-His_6 indicative of a physical interaction was determined by Western blot of the eluted sample. *B*, the same *in vitro* transcription and translation reactions were used to coexpress 3× FLAG epitope-tagged Swm1 with the indicated APC subunits containing a His_6 tag. Expression of each protein was confirmed by Western blot (*Input*). Swm1-FLAG was purified using anti-FLAG affinity resin and eluted with 3× FLAG peptide, and the presence of co-purifying APC subunits was detected by Western blot with anti-His_6 antibody. There was no detectable expression of Apc4-His_6 in this experiment. *C*, Apc10-His_6, Cdc23-His_6, and Apc5-His_6 were coexpressed with Swm1-FLAG or expressed alone. Reactions were subjected to the same anti-FLAG affinity purification and elution performed in *A* and *B*. Expression of each subunit (*Input*) and the presence of the His_6-tagged proteins in the elution were monitored by Western blot with anti-His_6 antibody. To conserve space, only the full-length FLAG-tagged proteins are displayed in *A* and *B* to confirm expression, although in many cases numerous unfinished translation products or proteolytic fragments were also visible on the Western blots because the 3× FLAG epitope is present at the N terminus of each subunit. Therefore, the relative expression levels of each FLAG-tagged protein cannot be accurately compared from this *figure*. Also, it is important to note that the intensity of bands in the anti-His_6 *versus* anti-FLAG Western blots cannot be used to compare protein quantity. Therefore, there is no reliable way to compare the actual amount of each of the two subunits produced in a given reaction. Although not shown, we reprobed all gels containing the purified samples with anti-FLAG antibody to ensure that the immunoaffinity purifications were successful.

strain (Fig. 4*C* and Table II), consistent with the delayed anaphase entry reported previously for *apc9*Δ (7). The *mnd2*Δ strain exhibited an accumulation of G_2/M cells that was comparable in magnitude with the *apc9*Δ strain, suggesting that it too has a similar delay in mitotic progression. The accumulation of G_2/M cells in the *apc10*Δ strain was more pronounced than that of *apc9*Δ or *mnd2*Δ, although perhaps not as much as would be expected given its severely retarded growth. Perhaps the lack of Apc10 impairs progression through G_1 as well as M phase or has an independent effect on overall cell growth. Finally, the *swm1*Δ strain exhibited a dramatic increase in the percentage of G_2/M cells compared with the wild-type parent strain (63% *versus* 29%, Table II). The greater coefficients of variation for the G_1 and G_2/M peaks of *apc10*Δ and *swm1*Δ (Fig. 4*C*) as well as their forward scatter data (not shown) suggest significant morphological changes in these strains compared

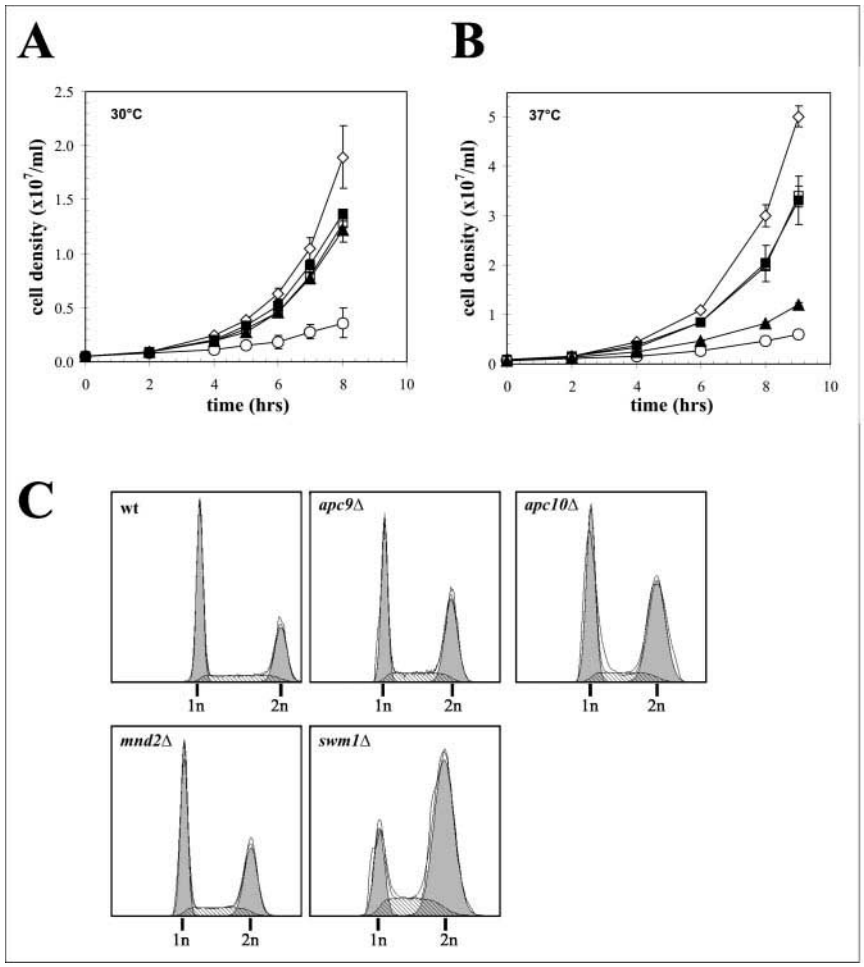

FIG. 4. **Effect of *MND2* and *SWM1* deletions on yeast cell growth and the cell cycle.** Relative growth rates of haploid yeast strains harboring deletions of *APC9* (■), *APC10* (○), *MND2* (□), and *SWM1* (▲) and their isogenic parent strain BY4741 (◇) were monitored at 30 °C (*A*) or 37 °C (*B*), starting from identical cell densities. Three independent cultures of each strain were used in a single experiment. The *graphs* depict a typical single experiment, with data points representing the average of the three cultures. The *error bars* indicate S.D. *C*, samples from each strain grown at 37 °C (*B*) were removed at OD_{660} = 0.5 and analyzed by flow cytometry as described under "Experimental Procedures." ModFit LT analysis software was used to extract the percentages of cells from each strain in G_1, S, and G_2/M phases. *Solid areas* represent the computer-generated fit for G_1 (*1n*) and G_2/M (*2n*), and the *striped area* represents the fit for S phase. The numerical values for the G_2/M peaks from the computer fitting are displayed in Table II.

TABLE II
Quantitative analysis of G_2/M content from wild-type and deletion strains

All strains are isogenic with the exception of the noted gene deletions. Percentages represent averages and S.D. values from three cultures grown at 37 °C that are depicted in Fig. 4, *B* and *C*. Data values were generated by ModFit LT flow cytometry analysis software.

Strain	G_2/M
	%
Wild type	29.2 ± 0.7
*apc9*Δ	40.0 ± 0.6
*apc10*Δ	46.2 ± 3.4
*mnd2*Δ	36.2 ± 0.9
*swm1*Δ	63.0 ± 0.8

with the parental strain. Considering our convincing evidence that Swm1 is a component of the APC, it is reasonable to conclude that *swm1*Δ cells have a substantial delay in progression through mitosis resulting from an APC defect that is reflected in their slow growth and dramatic accumulation of G_2/M cells illustrated in Fig. 4. Although we cannot rule out the possibility that Mnd2 and/or Swm1 have APC-independent cellular functions that affect vegetative growth and progression of cells through G_2 and M phase, our results are consistent with the conclusion that Mnd2 and Swm1 are constitutive components of the yeast APC that contribute to normal APC activity during mitosis.

DISCUSSION

In this report, we have provided strong evidence that Mnd2 and Swm1 are constitutive core subunits of the budding yeast APC. Swm1 is probably equivalent to a 19-kDa protein labeled Apc13 that was previously observed in an APC immunoaffinity purification (7) but not identified at the time. These findings bring the total number of confirmed subunits in budding yeast APC to 13. Our ability to identify two previously unidentified subunits of the APC emphasizes the power of mass spectrometry as a sensitive and accurate analytical tool for biological research and the utility of the 3× FLAG epitope for immunoaffinity purification of protein complexes. Another group recently achieved the same identification of Mnd2 and Swm1 by coupling mass spectrometry and the tandem affinity purification method (20). Our protocol allows a rapid one-step preparation that can be completed in a single day and provides high yield and purity as evidenced by the lack of nonspecific contaminants and the ability to see proteins by Coomassie staining in Fig. 1*A*. Further advantages of the FLAG-based immunoaffinity purification include the small size of the 3× FLAG epitope (less than 3 kDa) compared with other commonly used affinity tags, making it less likely to impair normal protein function, and the lack of specific buffer requirements, which, for example, allows the purification to be performed at high salt concentrations if desired.

In addition to identifying Mnd2 and Swm1 as components of the APC throughout the cell cycle, we have defined the subunit-subunit interactions that mediate Mnd2 and Swm1 association with the complex using a powerful interaction assay based on an *E. coli in vitro* transcription and translation system (Fig. 3). A key advantage of the *E. coli*-based system is the lack of endogenous APC subunit homologs that can potentially interfere with interaction assays using eukaryotic expression systems. In identifying the subunit contacts of Mnd2 and Swm1, we have taken an important first step in defining the organi-

zation of the complex that will be important for understanding the structure and function of the APC.

We have demonstrated that *mnd2*Δ and *swm1*Δ haploid strains have mitotic phenotypes consistent with an APC defect, including retarded growth rates and accumulation of G_2/M cells (Fig. 4). Although these data suggest a significant contribution of Mnd2 and Swm1 to the mitotic function of the APC, they are clearly not essential as are the majority of APC subunits. This is in contrast to diploid yeast in which Mnd2 and Swm1 are essential for progression through meiosis. An *mnd2*Δ diploid strain arrests prior to meiotic nuclear division (16), and a *swm1*Δ diploid strain arrests late in meiosis prior to spore wall formation (15). The different requirement for Mnd2 and Swm1 in mitosis *versus* meiosis has interesting implications for how the APC functions and why it has so many subunits. There are two possible general explanations for the difference in mitotic and meiotic phenotypes. The most likely is that Mnd2 and Swm1 provide an essential function for the APC during meiosis that is not required during mitosis. A second possibility that currently lacks supporting evidence is that Mnd2 and Swm1 perform essential APC-independent functions during meiosis.

Because most of the APC subunits are essential for viability, the separation of function within the APC suggested by the *mnd2*Δ and *swm1*Δ mitotic and meiotic phenotypes has not been previously described. Furthermore, the role of APC in meiosis has not received as much attention as its role in mitosis. Specific functions for APC subunits during meiosis could include recruitment of meiosis-specific substrates, activation or inhibition of APC toward specific substrates in response to meiosis-specific signals, or mediating cellular localization required for proper meiotic function of APC. Budding yeast APC is known to interact with a meiosis-specific substrate activator protein, Ama1 (21). Perhaps Mnd2 or Swm1 are involved in the binding of Ama1 or recruitment of Ama1-specific substrates during meiosis. Similarly, a fission yeast protein, Mfr1, was identified as an activator of APC during meiosis that coordinates nuclear division and sporulation (22), and a meiosis-specific kinase in budding yeast has also been identified as a negative regulator of APC activity (23). It is conceivable that Mnd2 or Swm1 might be involved in the interaction of APC with these proteins as well. Future research will hopefully provide insight into details of APC function in mitosis *versus* meiosis, and it may also prove interesting to examine possible specific functions for the other nonessential APC subunits Apc9, Apc10, and Cdc26 in meiosis.

A specific function for Mnd2 and Swm1 during meiosis raises a question about the nature of their nonessential role in mitosis. The phenotype of *mnd2*Δ haploid yeast is mild, and it is possible that lack of Mnd2 in mitosis affects the stability of the complex enough to indirectly impair catalysis or some other property of the APC. Similarly, the slow growth phenotype of *swm1*Δ haploid yeast is severe at 37 °C but mild and comparable with *mnd2*Δ at 30 °C, consistent with a defect in complex stability that would be exacerbated at elevated temperatures. This is similar to the Cdc26 subunit that has been suggested to aid in complex stability at elevated temperatures because it is required for growth at 37 °C but seems to be almost entirely unnecessary for normal growth at 30 °C (5). Alternatively, Mnd2 and Swm1 function may be required specifically for the polyubiquitination of a substrate or set of substrates whose degradation is essential for progression through meiosis but plays only a minor role in mitosis. Analysis of substrate-specific ubiquitin ligase activity of APC lacking Mnd2 or Swm1 might help determine whether their presence is required specifically for certain substrates or contributes generally in some manner to basic APC activity.

An APC-independent function for Mnd2 and Swm1 during meiosis remains a formal possibility. This would probably require the existence of free Mnd2 and Swm1 after initiation of sporulation. *MND2* expression is moderately up-regulated in meiosis (16, 24), whereas *SWM1* expression is greatly increased (15, 24). However, most of the APC subunits demonstrate a sharp increase in expression during meiosis as well (19, 24), suggesting that the levels of the entire complex are increased. The reason for increased expression of the APC during meiosis is not known. We examined the components of meiotic APC after purification of the complex from sporulating diploid cells and found that Mnd2 and Swm1 remain associated with the APC during meiosis. This result is more consistent with the notion that an APC-specific defect is responsible for the severe meiotic phenotypes in strains lacking functional Mnd2 and Swm1.

Mnd2 and Swm1 do not have any obvious mammalian homologs (15) (data not shown). Weak homology to subunits of the *S. pombe* APC was recently suggested (20), but the lack of strong homology suggests the possibility that different organisms may contain species-specific APC subunits. This possibility seems unlikely at first, given the fact that the APC is essential for execution of some of the most fundamental cellular processes common to all eukaryotic cells. It is possible that during evolution, species have been able to recruit the ubiquitin ligase activity of the APC to function in other types of cellular transactions that are not necessarily highly conserved. Aside from Mnd2 and Swm1, budding yeast Apc9 (7) appears to have no homolog in other organisms, and metazoans contain a subunit, APC7 (9), that does not appear to have a homolog in yeast. Perhaps the large number of subunits reflects, in part, a large number of biological roles in which the APC acts as a type of modular enzyme complex with a catalytic core involving Apc2 and Apc11 and a host of other subunits to direct the ubiquitin ligase activity to specific substrates at specific times and locations within the cell. Another ramification of the identification of Mnd2 and Swm1 is the likelihood that other subunits might still be unidentified in other organisms such as humans. A major focus of future research will no doubt be focused on identification of other APC subunits in higher eukaryotes and understanding the roles of the numerous APC subunits that to this date have no known functions.

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