The *Schizosaccharomyces pombe* *dim1*⁺ Gene Interacts with the Anaphase-Promoting Complex or Cyclosome (APC/C) Component *lid1*⁺ and Is Required for APC/C Function

LYNNE D. BERRY,¹ ANNA FEOKTISTOVA,² MELANIE D. WRIGHT,¹
AND KATHLEEN L. GOULD^{1,2*}

*Howard Hughes Medical Institute² and Department of Cell Biology,¹
Vanderbilt University School of Medicine, Nashville, Tennessee 37232*

Received 5 August 1998/Returned for modification 21 September 1998/Accepted 22 December 1998

The *Schizosaccharomyces pombe* *dim1*⁺ gene is required for entry into mitosis and for chromosome segregation during mitosis. To further understand *dim1*p function, we undertook a synthetic lethal screen with the temperature-sensitive *dim1*-35 mutant and isolated *lid* (for lethal in *dim1*-35) mutants. Here, we describe the temperature-sensitive *lid1*-6 mutant. At the restrictive temperature of 36°C, *lid1*-6 mutant cells arrest with a “cut” phenotype similar to that of *cut4* and *cut9* mutants. An epitope-tagged version of *lid1*p is a component of a multiprotein ~20S complex; the presence of *lid1*p in this complex depends upon functional *cut9*⁺. *lid1*p-myc coimmunoprecipitates with several other proteins, including *cut9*p and *nuc2*p, and the presence of *cut9*p in a 20S complex depends upon the activity of *lid1*⁺. Further, *lid1*⁺ function is required for the multiubiquitination of *cut2*p, an anaphase-promoting complex or cyclosome (APC/C) target. Thus, *lid1*p is a component of the *S. pombe* APC/C. In *dim1* mutants, the abundances of *lid1*p and the APC/C complex decline significantly, and the ubiquitination of an APC/C target is abolished. These data suggest that at least one role of *dim1*p is to maintain or establish the steady-state level of the APC/C.

The *cdc2* protein serine/threonine kinase plays a key role in promoting cell cycle progression in all eukaryotes (reviewed in references 30, 34, and 37). At the G₂/M transition, cells enter mitosis as a result of *cdc2*p activation, which in turn is dependent upon the association of *cdc2*p with its positive regulatory partner, cyclin B. The timed destruction of cyclin B at the end of mitosis is one mechanism ensuring that *cdc2*p-cyclin B is inactivated in each cell cycle.

Indeed, the abrupt disappearance of cyclin observed at the end of each cell cycle in fertilized sea urchin eggs first suggested the possibility that cyclin destruction may be important for cell cycle progression (10). The fact that nondegradable forms of cyclin B injected into frog egg cycling extracts prevented exit from mitosis further strengthened this hypothesis (35). It has recently been demonstrated that although cyclin B is normally degraded during mitosis in all eukaryotes to promote exit from mitosis (reviewed in references 8, 17, and 26), at least in budding yeast, the destruction of *Clb2*p, the major mitotic cyclin, is not absolutely required for cell cycle progression (42, 45).

Destruction of cyclins at mitosis occurs via ubiquitin-mediated proteolysis (14), a multistep process in which a ubiquitin-activating enzyme (E1) first activates ubiquitin by formation of a high-energy thioester bond. E1 then transfers ubiquitin to an E2 or ubiquitin-conjugating enzyme. The E2 may transfer ubiquitin directly to the substrate, targeting the substrate for destruction by the 26S proteasome. Alternatively, the specificity of this reaction may be determined by association of the E2 with a ubiquitin protein ligase (E3) to catalyze ubiquitination of a target protein (reviewed in references 7 and 19).

An E3 ubiquitin ligase that recognizes A- and B-type cyclins

was identified biochemically in clam and frog extracts and genetically in fission and budding yeasts and is known as the anaphase-promoting complex or cyclosome (APC/C) (reviewed in references 8, 17, 26, and 44). The APC/C is a multisubunit complex of ~20S that has been conserved throughout evolution (reviewed in references 8, 17, 26, and 44). In *Xenopus* egg extracts, eight components of the APC have been identified (27, 36), and the sequences of their human homologs have been reported (50). In *Saccharomyces cerevisiae*, 10 APC components have been identified (22, 23, 29, 51–53). In *Schizosaccharomyces pombe*, only four APC components have been identified to date: *cut4*p (49), *cut9*p (40, 47), *nuc2*p (18, 28), and *hcn1*p (47). *cut4*p, *cut9*p, and *nuc2*p are orthologs of *S. cerevisiae* *Apc1*p, *Cdc16*p, and *Cdc27*p and of human APC1, APC6, and APC3, respectively. *hcn1*p is related to *S. cerevisiae* *Cdc26*p and is apparently important for APC/C activity only at elevated temperatures (47). The *cut4*, *cut9*, and *nuc2* gene products interact physically, forming part of an ~20S complex (47, 49). Temperature-sensitive *cut4* and *cut9* mutants all display a “cut” phenotype at restrictive temperatures; in these mutants, chromosome segregation and spindle elongation fail to occur, such that subsequent cytokinesis bisects the nucleus or results in segregation of DNA to only one daughter cell (40, 49).

The APC/C targets proteins containing a destruction box motif for ubiquitin-dependent proteolysis during mitosis and G₁ phases. While M phase cyclins were the first targets known, other APC/C target proteins have subsequently been identified. Anaphase chromosome segregation in the budding yeast *S. cerevisiae* requires APC/C-mediated destruction of the nuclear protein *Pds1* (9, 48), and in the fission yeast *S. pombe*, chromosome segregation requires destruction of *cut2*p (12). Additional substrates of the APC/C include the spindle component *Ase1*p (24), cohesins (32), the *Cdc5*p “polo” kinase (6, 43), and *Cdc20*p (43). In the absence of APC/C activity, spindle

* Corresponding author. Mailing address: HHMI and Dept. of Cell Biology, Vanderbilt University School of Medicine, Nashville, TN 37232. Phone: (615) 343-9502. Fax: (615) 343-0723. E-mail: kathy.gould@mcmail.vanderbilt.edu.

TABLE 1. *S. pombe* strains used in this study

Strain	Genotype	Source or reference
KGy28	<i>h</i> [−] 972	P. Nurse
KGy42	<i>h</i> ⁺ <i>cdc25-22</i>	P. Nurse
KGy69	<i>h</i> ⁺ 975	P. Nurse
KGy396	<i>h</i> ⁺ <i>dim1-35 leu1-32</i>	5
KGy656	<i>h</i> [−] <i>cdc10-V50 leu1-32</i>	P. Nurse
KGy864	<i>h</i> [−] <i>nuc2-663</i>	P. Nurse
KGy1150	<i>h</i> [−] <i>lid1-6 ura4-D18 leu1-32 ade6-M210</i>	This study
KGy1162	<i>h</i> [−] <i>lid1-6 dim1-35 leu1-32</i>	This study
KGy1164	<i>h</i> [−] <i>cut9-665 leu1-32</i>	R. McIntosh
KGy1166	<i>h</i> [−] <i>lid1-6</i>	This study
KGy1177	<i>h</i> [−] <i>lid1-6 cut9-665 leu1-32 ade6-M216</i>	This study
KGy1180	<i>h</i> ⁺ <i>dim1::his3⁺ leu1-32::p980 his3-D1 ura4-D18 ade6-M210</i>	5
KGy1186	<i>h</i> [−] <i>cut4-533 leu1-32</i>	R. McIntosh
KGy1192	<i>h</i> ⁺ <i>lid1-6 cut4-533</i>	This study
KGy1203	<i>h</i> [−] <i>lid1-6 leu1-32::pKG1025 ura4-D18 ade6-M210</i>	This study
KGy1302	<i>h</i> ⁺ <i>lid1::lid1-myc/Kan^r</i>	This study
KGy1305	<i>h</i> [−] <i>lid1::lid1-myc/Kan^r dim1-35</i>	This study
KGy1335	<i>h</i> ⁺ / <i>h</i> [−] <i>lid1⁺/lid1::ura4⁺ ura4-D18/ura4-D18 leu1-32/leu1-32 ade6-M210/ade6-M216</i>	This study
KGy1336	<i>h</i> [−] <i>lid1::lid1-myc/Kan^r ura4-D18 leu1-32 ade6-M210</i>	This study
KGy1365	<i>h</i> ⁺ <i>cut9::cut9-HA/Kan^r</i>	This study
KGy1366	<i>h</i> [−] <i>lid1::lid1-myc/Kan^r cut9::cut9-HA/Kan^r</i>	This study
KGy1402	<i>h</i> [−] <i>cdc10-V50 lid1-6</i>	This study
KGy1404	<i>h</i> [−] <i>lid1::lid1-myc/Kan^r cut9-665</i>	This study
KGy1462	<i>h</i> [−] <i>cut9::cut9-HA/Kan^r lid1-6</i>	This study
KGy1561	<i>h</i> [−] <i>dim1::his3⁺ leu1-32::p980 his3-D1 ura4-D18 ade6-M210 lid1::lid1-myc/Kan^r</i>	This study
KGy1739	<i>h</i> [−] <i>dim1-35 cut9::cut9-HA/Kan^r</i>	This study
KGy1570	<i>h</i> [−] <i>cut2::cut2-myc Kan^r</i>	This study
KGy1571	<i>h</i> ⁺ <i>cut2::cut2-myc Kan^r</i>	This study
KGy574	<i>h</i> [−] <i>mts3-1 leu1-32</i>	C. Gordon
KGy1923	<i>h</i> [−] <i>mts3-1 cut2::cut2-myc Kan^r leu1-32</i>	This study
KGy948	<i>h</i> [−] <i>mts3-1 lid1-6 cut2::cut2-myc leu1-32 ura4-D18</i>	This study
KGy2035	<i>h</i> [−] <i>mts3-1 dim1-35 cut2::cut2-myc leu1-32 ura4-D18</i>	This study

elongation, chromosome segregation, and exit from mitosis are blocked.

Like components of the APC/C, dim1p also is required during mitosis for chromosome segregation (5). dim1p is a highly conserved 17-kDa protein whose biochemical function is unknown. In *S. cerevisiae*, the ortholog of *dim1*⁺ was originally called *CDH1* (5), but the name has been changed to *DIB1*. In an effort to further elucidate *dim1*⁺ function, we undertook a synthetic lethal screen with the temperature-sensitive *dim1-35* mutant and isolated *lid* (for lethal in *dim1-35*) mutants. Here, we describe the temperature-sensitive *lid1-6* mutant. At the restrictive temperature of 36°C, *lid1-6* mutant cells arrest with a cut phenotype similar to that of *cut4* and *cut9* mutants. An epitope-tagged version of lid1p (*lid1p-myc*) is present in a high-molecular-weight complex of ~20S, and the presence of lid1p in this complex depends upon functional cut9p. Coimmunoprecipitation analyses demonstrate an in vivo association among lid1p-myc and several other proteins, including cut9p and nuc2p, and the presence of cut9p in a 20S complex depends upon the activity of lid1p. Further, the multiubiquitination of cut2p depends upon *lid1*⁺ function. Thus, lid1p is a component of the *S. pombe* APC/C. To explain the genetic interaction between *dim1* and *lid1* mutants, we have found that the abundances of lid1p and the 20S APC/C complex are dependent upon *dim1*⁺ function. Moreover, *dim1* function is required for APC/C-mediated multiubiquitination of cut2p. These data indicate that one role of dim1p in mitosis is to establish or maintain the integrity and function of the APC/C.

MATERIALS AND METHODS

Yeast methods, strains, and media. The *S. pombe* strains used in this study are listed in Table 1. Strains were grown in yeast extract (YE) medium or minimal medium with appropriate supplements (33). Crosses were performed on malt extract medium (33) or glutamate medium (minimal medium lacking ammonium chloride and containing 0.01 M glutamate, pH 5.6). Random spore analysis and tetrad analysis were performed as described previously (33). Double-mutant strains were constructed and identified by tetrad analysis. Unless otherwise indicated, transformations were performed by electroporation (38). Genomic DNA was isolated as described previously (20, 33).

Plasmids and molecular biological techniques. All plasmid manipulations and bacterial transformations were by standard techniques (39). Essential features of plasmid construction are described below. All sequencing of plasmid DNA was performed by using Sequenase 2.0 (U.S. Biochemicals, Cleveland, Ohio) or Thermo Sequenase (Amersham Life Sciences, Cleveland, Ohio) according to the manufacturer's instructions. Unless otherwise specified, all PCRs were performed by using *Taq* DNA polymerase and the GeneAmp PCR reagent kit (Perkin-Elmer) in a PTC-100 Programmable Thermal Controller (MJ Research, Watertown, Mass.) programmed as follows: 94°C for 1 min, 50°C for 2 min, and 72°C for 2 min (40 cycles) and 72°C for 10 min. All [³²P]dCTP-labeled probes for radioactive hybridization were labeled by using *redivue* [³²P]dCTP (Amersham Life Sciences, Amersham, England) and the *rediprime* random primer labeling kit (Amersham Life Sciences) according to manufacturer's instructions.

***dim1-35* synthetic lethal screen (*lid* screen).** A *dim1-35* strain carrying plasmid-borne mouse *dim1* (*mdim1*) under control of the *nmt1* promoter, pKG930 (5), was mutagenized with nitrosoguanidine as described previously (33). Approximately 85,000 mutagenized cells were plated at a density of 300 to 500 cells per plate on medium lacking thiamine in order to induce expression of *mdim1*. Plates were incubated at 25°C until colonies formed. Colonies were replica plated to medium containing thiamine in order to eliminate *mdim1* activity. We used *mdim1* for this screen because *S. pombe dim1* activity under control of the *nmt1* promoter was not effectively eliminated by the inclusion of thiamine in the medium, whereas *mdim1* activity clearly was. Plates were incubated overnight at 25°C and then re-plated to medium containing thiamine. Plates were screened 2 days later for colonies that were inviable in the presence of thiamine.

Physiological experiments. For analysis of synchronous cell populations, 4 liters of cells was grown to mid-log phase (8 × 10⁶ cells/ml) at the permissive

temperature (25°C) in YE medium. Cells were separated on the basis of size by centrifugal elutriation in a Beckman JE 5.0 elutriator rotor. Cells synchronized in early G₂ (the smallest cells in the population) were collected and inoculated into YE medium at 25 or 36°C. Synchrony was monitored at 20- to 25-min intervals by scoring 100 cells for the presence of a septum.

Flow cytometry and microscopy. For flow cytometric analysis, cells were fixed in ice-cold 70% ethanol, washed in 50 mM sodium citrate, incubated with 0.1 mg of RNase A per ml in 50 mM sodium citrate for 2 h at 37°C, and then stained with 1 μ M Sytox green in 50 mM sodium citrate for 1 h. Cells were sonicated and analyzed by flow cytometry as described previously (41). All fluorescence microscopy was performed on a Zeiss Axioskop with appropriate filters. In order to visualize DNA, ethanol-fixed cells were washed in phosphate-buffered saline and then stained with the fluorescent DNA-binding dye DAPI (4',6-diamidino-2-phenylindole) at 1 μ g/ml. For immunofluorescence, cells were fixed in 70% ethanol at 4°C, or in 100% methanol at -20°C, for 8 min and then washed with phosphate-buffered saline and processed as described previously (2). For staining of microtubules, fixed cells were incubated in a 1:10 dilution of the monoclonal TAT-1 primary antibody (46) (a generous gift of K. Gull) followed by a 1:100 dilution of Texas red-conjugated goat antimouse secondary antibody (Molecular Probes, Eugene, Oreg.).

Cloning and sequence analysis of *lid1*. *lid1-6* mutant cells (KGY1150) transformed with a pUR19-based *S. pombe* genomic library (3) were selected at 25°C on medium lacking uracil and then replica plated to 36°C. Plasmids subsequently shown to be identical by restriction digestion and Southern blot analysis were recovered from two independent *Lid1*⁺ *Ura*⁺ colonies. Deletion constructs generated from the rescuing plasmid (pKG1030) were retransformed into *lid1-6* cells in order to identify a minimal rescuing fragment. Sequencing of pKG1030 revealed that a portion of the insert was contained within c19g12 (21), a cosmid sequenced as part of the *S. pombe* genome sequencing project. The remainder of the insert was sequenced and, in combination with sequence obtained from c19g12, analyzed for coding potential.

For integration mapping, the complete 5.5-kb pKG1030 insert was subcloned into the *S. pombe ura4*-based integrating vector pJK210 (25) to generate pKG1025. pKG1025 was linearized at the unique *SacI* site within the insert, and the linearized plasmid was transformed into the *lid1-6 ura4-D18* mutant strain KGY1150. Transformants were selected on medium lacking uracil. *Lid1*⁺ *Ura*⁺ transformants were picked and outcrossed to either *lid1*⁺ or *lid1-6* strains in order to verify cosegregation of the *lid1*⁺ phenotype with the *ura4*⁺ marker.

Isolation of *lid1* cDNAs. An *S. pombe* cDNA library constructed in the vector pDB20 (11) was transformed into *Escherichia coli* and plated on Luria-Bertani agar containing 50 μ g of ampicillin per ml. Approximately 10⁶ transformants were plated. Colonies were allowed to form overnight at 36°C and then lifted onto Hybond-N nylon membranes (Amersham Life Sciences) and processed for radioactive hybridization according to manufacturer's instructions. Membranes were prehybridized in 5 $\times$ Denhardt's solution-0.5% sodium dodecyl sulfate (SDS)-5 $\times$ SSPE (1 $\times$ SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA [pH 7.7])-100 μ g of hydrolyzed yeast RNA per ml at 65°C and then hybridized overnight in the same buffer. Two different templates were utilized in order to generate [³²P]dCTP-labeled probes: a 1.5-kb *lid1*⁺ *EcoRI* fragment (used to isolate clone pKG1074) and a 0.5-kb 5' *lid1*⁺ *SacI/XhoI* fragment (used to isolate pKG1142). The isolated plasmids were sequenced by using gene-specific oligonucleotide primers.

Deletion of *lid1* from the genome. In order to generate a *lid1* deletion construct containing *lid1*⁺ 5' and 3' flanks, an ~4-kb *lid1*⁺ genomic fragment (*BglII/BstXI*) was subcloned from pKG1030 into pBS-SK⁺ (Stratagene, La Jolla, Calif.) to generate pKG1186. A portion of the *lid1* coding region (*XhoI/EcoRV*) was excised from pKG1186 and replaced with a 1.8-kb fragment containing the *ura4*⁺ gene, to generate pKG1191. A *SacI/Acc651* fragment consisting of *lid1* 5' flank::*ura4*⁺::*lid1* 3' flank was isolated from pKG1191 and transformed into a diploid strain (*h*⁺/*h*⁻ *ura4-D18/ura4-D18 leu1-32/leu1-32 ade6-M210/ade6-M216*). *Ura*⁺ transformants were selected on medium lacking uracil. Uracil prototrophs were isolated, and genomic DNA was prepared and digested with *BglII* and *HindIII*. Digests were separated by agarose gel electrophoresis, blotted to membranes, and hybridized with an [α -³²P]dCTP-labeled 1-kb *BglII/XhoI* fragment of *lid1* 5' flank, in order to verify replacement of one allele of *lid1* with the *ura4*⁺ cassette in the *Ura*⁺ diploids. Diploids were allowed to sporulate, and tetrads were dissected in order to analyze the *lid1*::*ura4*⁺ phenotype.

Epitope tagging of *lid1*. A genomic version of *lid1* encoding nine copies of the myc epitope fused to the C terminus of *lid1* was generated by the method described previously (1). In brief, the oligonucleotide primers *lid1*tag1 (5'-CCT GTA TCT AGA TGC CAC TTT GGC-3') and *lid1*tag3 (5'-GGG GAT CCG TCG ACC TGC AGC GTA CGA AAA AGA GAA TAA CAG ATA TCT CG-3') were synthesized in order to PCR amplify a 400-bp *lid1* genomic fragment upstream of the putative *lid1* stop codon, and the primers *lid1*tag2 (5'-TTA TAT ATA AGG TAC CTT GAT TC-3') and *lid1*tag4 (GTT TAA ACG AGC TCG AAT TCA TCG ATA TGA GCT TAT GTT TAA TGG TCG-3') were synthesized in order to PCR amplify *lid1* genomic sequence downstream of the putative *lid1* stop codon, using the thermostable DNA polymerase *Pfu* (Stratagene) according to the manufacturer's instructions in a PTC-100 Programmable Thermal Controller (MJ Research) programmed as follows: 95°C for 1 min, 56°C for 1 min, and 72°C for 3 min (40 cycles); 94°C for 1 min; 56°C for 2 min; and 72°C for 10 min. PCR fragments were agarose gel purified by using the QIAquick

Gel Extraction System (QIAGEN) according to the manufacturer's instructions. Approximately 100 ng of each fragment was used in a second PCR, with a mixture containing 1 μ M each *lid1*tag1 and *lid1*tag2 and 200 ng of pKG1198 (encoding the 9xMyc-Kan tag; the 9xMyc cassette was a generous gift of K. Nasmyth, and pKG1198 was constructed by W. H. McDonald) and utilizing the Taq Plus Precision System (Stratagene) according to the manufacturer's instructions, in a PTC-100 Programmable Thermal Controller (MJ Research) programmed as follows: 95°C for 1 min; 95°C for 1 min, 62°C for 1 min (with an increase of 1°C/cycle), and 72°C for 5 min (15 cycles); 95°C for 1 min, 50°C for 1 min, and 72°C for 5 min (30 cycles); and 72°C for 10 min. The amplification product was agarose gel purified and transformed into diploid cells (*h*⁺/*h*⁻ *ura4-D18/ura4-D18 leu1-32/leu1-32 ade6-M210/ade6-M216*) by the lithium acetate method, as described previously (25). Transformants were plated on YE agar, allowed to recover overnight at 32°C, and then replica plated to YE agar containing 25 μ g of G418 per ml. G418-resistant (G418^r) diploids were picked and allowed to sporulate. Tetrads were dissected and analyzed for segregation of the G418^r marker. G418^r colonies derived from tetrads which segregated 2 G418^r:2 G418^s progeny were picked and further analyzed. Genomic DNA was prepared and digested with *SalI* and *NdeI*. Digests were separated by agarose gel electrophoresis, blotted to membranes, and hybridized with an [α -³²P]dCTP-labeled 600-bp *HindIII lid1* fragment, in order to verify integration of the *lid1* tag at the *lid1* genomic locus in G418^r colonies. Protein lysates were prepared as described previously (16) and subjected to immunoprecipitation by consecutive incubation with (i) the monoclonal anti-Myc antibody 9E10 (2 μ g/ml), (ii) rabbit anti-mouse immunoglobulin G (IgG) antibody (Organon Teknika Corp., West Chester, Pa.), and (iii) protein A-Sepharose (Pharmacia Biotech). Immunoprecipitates were washed extensively with Nonidet P-40 (NP-40) buffer. Lysates and immunoprecipitates were resolved on SDS-6 to 20% polyacrylamide gradient gels and then transferred to Immobilon-P (Millipore, Bedford, Mass.). For detection of *lid1*-myc, the blot was incubated consecutively with 9E10 (2 μ g/ml), peroxidase-conjugated sheep anti-mouse IgG (1:3,000; Sigma Chemical Co., St. Louis, Mo.), and enhanced chemiluminescence (ECL) detection reagents (Amersham Life Sciences), followed by chemiluminescence detection with Reflection NEF-596 autoradiography film (NEN Life Science Products).

Epitope tagging of cut9p. Epitope tagging of Cut9 was performed as described above for epitope tagging of *lid1*, using the 5' flank oligonucleotide primers *cut9*tag1 (5'-GAT GCC CTT AAC CAA GGG-3') and *cut9*tag2 (5'-GGG GAT CCG TCG ACC TGC AGC GTA CGA TCG TTG CTC TGA GAC ATT ACC TTC-3') and the 3' flank oligonucleotide primers *cut9*tag3 (5'-GTT TAA ACG AGC TCG AAT TCA TCG ATA TTG CGA AAT TCT ATT AAT TCT TG-3') and *cut9*tag4 (5'-GAA TTC TGC CGC TTC TAT G-3'). The second PCR was performed with pKG1155 (encoding the 3xHA-Kan tag; a generous gift of J. Bahler and J. Pringle) as the template. The purified amplification product was transformed directly into haploid wild-type cells (KGY28). G418^r colonies were picked and analyzed. Genomic DNA was prepared and digested with *BamHI*. Digests were separated by agarose gel electrophoresis, blotted to membranes, and then probed with the *cut9* 5' flank PCR fragment to verify integration of the 3xHA-Kan construct at the *cut9* locus. Protein lysates were prepared as described above and subjected to immunoprecipitation by consecutive incubation with (i) the monoclonal antihemagglutinin (anti-HA) antibody HA.11 (5 μ g/ml; Berkeley Antibody Company, Richmond, Calif.) or the monoclonal anti-HA antibody 12CA5 (5 μ g/ml), (ii) rabbit anti-mouse IgG antibody (Organon Teknika Corp.), and (iii) protein A-Sepharose (Pharmacia Biotech). Immunoprecipitates were washed extensively with NP-40 buffer. Lysates and immunoprecipitates were resolved on SDS-6 to 20% polyacrylamide gradient gels and then transferred to Immobilon-P (Millipore). For detection of cut9p-HA, the blot was incubated consecutively with HA.11 or 12CA5 (5 μ g/ml), peroxidase-conjugated sheep anti-mouse IgG (1:3,000; Sigma Chemical Co.), and ECL detection reagents (Sigma Chemical Co.), followed by chemiluminescence detection with Reflection NEF-596 autoradiography film (NEN Life Science Products). For quantitation of immunoblotting data, ECL Plus reagents (Amersham Life Science) were used. Data were collected on a Molecular Dynamics Storm instrument and quantified by ImageQuant version 1.1.

Epitope tagging of cut2p. Epitope tagging of cut2p was performed as described above for epitope tagging of *lid1*, using the 5' flank oligonucleotide primers *cut2*tag1 (5'-CAC CTG CAT CAG ATT TCC-3') and *cut2*tag3 (5'-GTT TAA ACG AGC TCG AAT TCA TCG ATA AAA GAT TAC GAA TTT TCA GGT TTT GTG-3') and the 3' flank oligonucleotide primers *cut2*tag2 (5'-GGG GAT CCG TCG ACC TGC AGC GTA CGA TAA CAA TCC TGT ATC CAA AGA TG-3') and *cut2*tag4 (5'-GAA TGT GTT GAT CAG CTG CTG-3'). The second PCR was performed with pKG1262 (encoding 13 copies of the myc epitope; from J. Bahler and J. Pringle) as the template. The purified amplification product was transformed directly into haploid cells (KGY28). G418^r colonies were picked and analyzed for the correct integration event by PCR.

In vivo ³⁵S labeling. Wild-type cells (KGY28) or *lid1*::*lid1*-myc cells (KGY1302) were grown to mid-log phase in minimal medium at 32°C. A total of 2 $\times$ 10⁸ cells were collected by centrifugation, resuspended in 10 ml of minimal medium containing 1 mCi of Tran³⁵S-label (ICN Pharmaceuticals, Inc., Irvine, Calif.), and then incubated with vigorous shaking at 32°C for 4 h. Cell lysates were prepared, and immunoprecipitation of *lid1*-myc was performed as described above. Immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 6 to 20% gradient gels. Gels were treated with

Amplify (Amersham Life Sciences), dried on a vacuum gel dryer, and exposed to X-ray film at -80°C .

Sucrose gradient analysis. For sucrose gradient analysis, cells were grown to mid-log phase in YE medium at 32°C . Approximately 2×10^8 cells were collected by centrifugation, and native lysates in NP-40 buffer were prepared as described above. Lysates were layered on 10 to 30% sucrose gradients prepared in NP-40 buffer. Gradients were ultracentrifuged at 30,000 rpm for 21 h in a Beckman SW50.1 rotor. Sedimentation markers were fractionated on gradients prepared and spun in parallel. Fractions were collected, run on SDS-6 to 20% polyacrylamide gradient gels, and then immunoblotted as described above.

Coimmunoprecipitation analysis of lid1p-myc and cut9p-HA. Native lysates were prepared from KGY1302 (*lid1::lid1-myc*), KGY1365 (*cut9::cut9-HA*), or KGY1366 (*lid1::lid1-myc cut9::cut9-HA*) cells as described above. Denatured lysates were prepared as for native lysates, except that lysed cells were heated to 100°C for 2 min in 300 μl of SDS lysis buffer (10 mM NaPO_4 [pH 7.0], 1% SDS, 1 mM dithiothreitol, 1 mM EDTA, 50 mM NaF, 100 μM Na_3VO_4 , 4 μg of leupeptin per ml) prior to extraction with NP-40 buffer. Immunoprecipitations were performed with 9E10 or HA.11 as described above. Immunoprecipitates were resolved by SDS-PAGE, blotted to polyvinylidene difluoride (PVDF) membranes, and probed for lid1p-myc or cut9p-HA as described above.

Ubiquitination assay. To assay APC/C activity towards cut2p-myc, we followed procedures outlined by Benito et al. (4). The relevant strains were transformed with pREP1-His₆-ubiquitin plasmid (a gift from Sergio Moreno). Following growth in the absence of thiamine for 22 h, cells were shifted to the nonpermissive temperature for 4 h and protein lysates were prepared as described previously (4). Briefly, cells were lysed in 8 M urea–100 mM sodium phosphate (pH 8.0)–5 mM imidazole. Cell extracts were clarified by centrifugation for 5 min, and the protein concentration in the supernatants was determined. Six milligrams of extract was mixed with Ni^{2+} -nitrilotriacetic acid (Ni^{2+} -NTA) agarose (Qiagen) and incubated for 4 h at room temperature. The resin was washed exactly as described previously (4). The eluate was diluted in 5 $\times$ sample buffer and boiled for 5 min, and ubiquitinated forms of cut2p-myc were detected by immunoblotting with 9E10 antibody as described above.

Analysis of protein levels through the cell cycle. A synchronized population of KGY1366 cells was isolated by centrifugal elutriation and then inoculated into fresh medium at 32°C . Approximately equivalent numbers of cells were collected at 20-min intervals for preparation of whole-cell lysates as described above. Lysates were resolved by SDS-PAGE and immunoblotted for cut9p-HA, lid1p-myc, and cdc13p, as described above, and for arp3p as a loading control. Antibodies to arp3p (31) were utilized at a dilution of 1:5,000, and affinity-purified rabbit anti-Cdc13 antibodies (GJG56) were used at a dilution of 1:500. Detection was as described above for lid1p-myc, except that peroxidase-conjugated sheep anti-rabbit IgG (1:10,000; Sigma Chemical Co.) was used as the secondary antibody. cdc2p was detected on immunoblots by incubation with anti-PSTAIRE monoclonal antibody (1:1,000; Sigma Chemical Co.), further incubation with peroxidase-conjugated goat anti-mouse IgG (1:50,000; Jackson ImmunoResearch laboratories, Inc.), and then ECL.

RESULTS

Isolation of *dim1*-interacting genes: the *lid* screen. As described previously, the *dim1* gene was isolated and shown to encode an evolutionarily conserved protein essential for mitosis (5). In order to investigate further the nature of *dim1* function, we wished to identify other proteins with which dim1p interacts in *S. pombe* cells. We took a genetic approach to this problem, choosing to isolate and characterize second-site mutations synthetically lethal with *dim1-35*. To accomplish this, a *dim1-35* strain carrying pREP1-*mdim1* was mutagenized and plated on medium lacking thiamine in order to induce expression of *mdim1*. Colonies were then replica plated to medium containing thiamine in order to eliminate *mdim1* activity. Under these conditions, we expected *dim1-35 lid* (for lethal in *dim1-35*) double mutants to be inviable. A total of 85,000 mutagenized colonies were screened; 88 were picked based on inviability in the presence of thiamine. Of these, 26 were outcrossed. Upon outcrossing, four of the mutant strains could not be analyzed due to poor spore viability. Based upon linkage between *dim1-35* and the *lid* mutation in each case, 10 of the remaining 22 strains appeared to contain unconditional loss-of-function alleles of *dim1*, while 12 contained second-site *lid* mutations unlinked to the *dim1* locus. Of the *lid* mutants, only the *lid1-6* mutant demonstrated a temperature-sensitive phenotype when outcrossed from the *dim1-35* mutant. Therefore, we chose to focus our efforts on the analysis of *lid1*.

***dim1*-interacting gene *lid1*.** *lid1-6* did not display strict synthetic lethality in a *dim1-35* background. When the outcrossed *lid1-6* mutant was crossed against the *dim1-35* mutant, viable *lid1-6 dim1-35* double mutants were recovered. The double-mutant cells, however, did display a near-lethal phenotype at either 25 or 29°C , temperatures fully permissive for either *lid1-6* or *dim1-35* single mutants (Fig. 1A). The double mutant grew extremely slowly and exhibited numerous cytological defects, including cutting and segregation of DNA to only one daughter cell (Fig. 1B, panel a), whereas either *lid1-6* or *dim1-35* single mutants were phenotypically wild type at these temperatures (Fig. 1B, panels b and c).

As mentioned above, the *lid1-6* mutant was unique among the *lid* mutants in displaying a lethal temperature-sensitive phenotype in a *dim1*⁺ background. At the restrictive temperature of 36°C , *lid1-6* single-mutant cells arrested with a cut phenotype, similar to that of *cut4* and *cut9* mutants (40, 49). In order to examine the arrest phenotype caused by *lid1-6* more carefully, *lid1-6* cells were synchronized in G₂ by centrifugal elutriation and then released into rich medium at a restrictive temperature. At the first mitosis, a minority of cells ($\sim 20\%$) completed mitosis successfully, as judged by antitubulin immunofluorescence and DAPI staining. The majority of cells ($\sim 80\%$), however, formed short mitotic spindles but failed to elongate the spindle and segregate their DNA. Subsequent septation resulted in development of a cut phenotype or missegregation of DNA to only one daughter cell (Fig. 1C to E). At the second mitosis, all cells exhibited the mutant phenotype. No elongated spindles were observed, and no cells completed mitosis successfully (Fig. 1C and D).

The similarity of the temperature-sensitive arrest phenotype of the *lid1-6* mutant to that of *cut4* and *cut9* mutants suggested the possibility that lid1p, like cut4p and cut9p, might comprise one component of the APC/C. Because genetic interactions often reflect protein-protein interactions, double mutants with *lid1* and either *cut4*, *cut9*, or *nuc2* (encoding a third component of the APC/C) mutations were constructed and analyzed. No genetic interactions between *lid1* and *cut9* or between *lid1* and *cut4* were detected. In contrast, the *lid1-6 nuc2-663* double mutant exhibited a lowering of restrictive temperature compared to either single mutant alone (data not shown).

Cloning and sequencing of *lid1*. In order to analyze *lid1* at a molecular genetic level, the *lid1* gene was cloned by complementation of the *lid1-6* temperature-sensitive phenotype. After transformation of the *lid1-6* mutant with an *S. pombe* genomic library, two rescuing plasmids, each containing a 5.5-kb insert, were isolated (pKG1030). The isolated plasmids were shown to be identical by restriction mapping and Southern blot analyses. Integration mapping confirmed that the isolated gene contained the *lid1* gene and not a high-copy suppressor. Sequencing revealed that a portion of the insert was contained within c19g12, a cosmid located on the short arm of chromosome I (21), which was sequenced as part of the *S. pombe* genome sequencing project. Sequencing of the remainder of the insert, in combination with the c19g12 sequence, revealed a single large open reading frame (ORF). The minimal rescuing fragment, however, extended beyond this ORF, suggesting that the gene included several smaller upstream ORFs, separated by introns (Fig. 2A). Therefore, a cDNA library was screened in order to isolate a *lid1* cDNA. Two independent clones, one a partial cDNA at the 3' end of the gene, containing the putative stop codon, and one a partial cDNA at the 5' end of the gene, were isolated. The 5' clone revealed the existence of four introns. Analysis of the genomic clone revealed an in-frame start codon 124 bp upstream of the first intron. Together, then, the isolated clones comprised an ORF (Fig. 2A) encoding a

FIG. 1. *lid1-6* mutant phenotype. (A) *lid1-6* and *dim1-35* display a near-lethal synthetic interaction. *lid1-6*, *dim1-35*, or *lid1-6 dim1-35* cells were streaked to YE agar and then incubated at 25 or 29°C for 3 days. (B) *lid1-6 dim1-35* (panel a), *lid1-6* (panel b), or *dim1-35* (panel c) cells were grown at 25°C, fixed, and stained with DAPI. (C to E) A synchronous population of *lid1-6* cells shifted to a restrictive temperature fails to undergo spindle elongation or chromosome segregation at the first mitosis. Subsequent septation results in missegregation of DNA or development of a cut phenotype. Cells were synchronized in early G₂ by centrifugal elutriation and then inoculated into rich medium at 36°C. Samples were collected at 20-min intervals and subjected to various analyses. (C) Spindle index and septation index of *lid1-6* synchronous cells after the shift. (D) Phenotypes of *lid1-6* cells after the shift. Note that at the first mitosis, a small percentage of cells undergo a normal anaphase (△), as evidenced by normal DNA segregation observed by DAPI staining. In the majority of cells, however, normal anaphase is not observed. Rather, cells cut or missegregate their DNA (■ and □). (E) DAPI (panel a) and tubulin (panel b) staining at 120 min after the shift (corresponding to the first spindle index peak) (note the abundance of short mitotic spindles and the absence of elongated spindles or normally segregated DNA) and DAPI (panel c) and tubulin (panel d) staining at 140 min after the shift (corresponding to the first peak of septation) (note that the majority of septated cells have cut or missegregated their DNA).

predicted protein product of 719 amino acids and 82.5 kDa (Fig. 2B). Comparison of the predicted amino acid sequence of lid1p with protein sequences available in the databases revealed no significant homologies or motifs (but see Discussion).

Deletion of *lid1* from the *S. pombe* genome. In order to determine whether *lid1* is an essential gene in *S. pombe*, the method of one-step gene disruption was used in order to replace a 3-kb fragment of DNA within the *lid1* minimal rescuing fragment with the *ura4⁺* selectable marker in diploid *S. pombe* cells (see Materials and Methods) (Fig. 2A). Replacement of

FIG. 2. Cloning of *lid1⁺*. (A) Restriction map of the *lid1⁺* genomic locus. Shown below the restriction map is the *lid1⁺* coding region. ■, intron; *, stop codon. Shown at bottom is the deletion construct. (B) DNA sequence and predicted amino acid sequence of *lid1⁺*. Intron sequences are in lowercase letters. *, stop codon.

one allele of *lid1* with the *ura4⁺* cassette in *Ura⁺* diploids was confirmed by Southern blot analysis (data not shown). *Ura⁺* diploids were allowed to sporulate, and tetrads were dissected. Thirty-five tetrads segregated 2 inviable:2 viable *Ura⁻* progeny. The inviable progeny underwent spore germination, and then two or three residual cell divisions, before arresting as microcolonies of six to eight cells (data not shown). Therefore, *lid1* encodes an essential gene in *S. pombe*.

lid1p-myc associates with several other proteins in vivo. In order to investigate *lid1* function at a biochemical level, a DNA fragment encoding nine copies of the myc epitope tag was

FIG. 3. lid1p-myc associates with several other proteins in vivo. (A) lid1p-myc is an ~100-kDa protein. KGY1302 whole-cell lysates (lane 1) or 9E10 immunoprecipitates from KGY1302 lysates (lane 2) were resolved by SDS-PAGE and transferred to a PVDF membrane, and the membrane was probed with the 9E10 antibody. Molecular mass standards (in kilodaltons) are indicated. (B) Wild-type (lanes 1 and 3) or KGY1302 (*lid1::lid1-myc*) (lanes 2 and 4) native ³⁵S-labeled cell lysates were immunoprecipitated with the 9E10 antibody, and immunoprecipitates were resolved by SDS-PAGE. Labeled proteins from two separate experiments for 1 day (lanes 1 and 2) or 7 days (lanes 3 and 4) were visualized by fluorography. Migrations of molecular mass markers (in kilodaltons) are indicated. Bands specific to the KGY1302 immunoprecipitate are indicated (●). An arrowhead indicates the band corresponding to lid1p-myc. (C) Cut9p-HA is an ~90-kDa protein. A KGY1365 whole-cell lysate (lane 1) or an HA.11 immunoprecipitate (lane 2) was resolved by SDS-PAGE and transferred to a PVDF membrane, and the membrane was probed with the HA.11 antibody.

integrated at the *lid1* genomic locus, just upstream of the putative *lid1* stop codon (Fig. 2) (see Materials and Methods). The anti-myc monoclonal antibody 9E10 detected a protein which migrated with an apparent molecular mass of ~100 kDa in lysates and 9E10 immunoprecipitates prepared from the epitope-tagged strain (*lid1::lid1-myc*; KGY1302) (Fig. 3A). This molecular mass corresponded to that predicted from the addition of the multiple myc epitope tags to the endogenous 82.5-kDa protein. This protein was not detected when lysates or 9E10 immunoprecipitates from wild-type cells were probed with the 9E10 antibody (data not shown).

In order to determine whether lid1p-myc associates with other proteins in vivo, *lid1-myc* or wild-type cells were labeled in vivo with Tran³⁵S-label. Whole-cell lysates were prepared under native conditions from labeled cells and then subjected to immunoprecipitation with the 9E10 antibody. Several proteins, including lid1p-myc, were immunoprecipitated from KGY1302 cell lysates but not from wild-type cell lysates, suggesting that lid1p is part of a multiprotein complex (Fig. 3B). That the indicated band corresponded to lid1p-myc was determined by 9E10 immunoprecipitation from ³⁵S-labeled denatured cell lysates (data not shown).

lid1-myc cosediments with cut9-HA. The specific, in vivo association of lid1p-myc with proteins of ~160, ~75, and ~65 kDa (Fig. 3B) was consistent with the hypothesis that lid1p may comprise a component of the APC/C in *S. pombe*. (APC/C

components cut4p, cut9p, and nuc2p are proteins of 166, 78, and 67 kDa, respectively [18, 40, 49]). In order to examine further the possibility that *lid1* may encode an APC/C component, an epitope-tagged version of the APC/C component cut9p was generated by integrating a DNA fragment encoding three copies of the HA epitope into the *S. pombe* genome just upstream of the *cut9* stop codon (see Materials and Methods). Lysates from the epitope-tagged strain (*cut9::cut9-HA*; KGY1365) were subjected to immunoprecipitation and immunoblot analysis with the monoclonal anti-HA antibody HA.11. A protein which migrated with an apparent molecular mass of ~90 kDa was detected in KGY1365 lysates and HA.11 immunoprecipitates (Fig. 3C) but not in wild-type lysates or immunoprecipitates (data not shown).

In order to determine whether lid1p cosediments with the APC/C, the sedimentation profiles of lid1p-myc and cut9p-HA were compared. A lysate from a double-tagged strain (*lid1::lid1-myc cut9::cut9-HA*) was prepared and subjected to sucrose gradient sedimentation analysis. Gradient fractions were collected, resolved by SDS-PAGE, blotted to membranes, and probed with either the anti-HA monoclonal antibody 12CA5 to detect cut9p-HA or the anti-myc monoclonal antibody 9E10 to detect lid1p-myc. lid1p-myc peaked three times: once in fraction 6, again in fraction 10, and lastly in fraction 15, which would correspond to monomeric lid1p-myc (Fig. 4A, panel a). cut9p-HA showed a bimodal distribution (Fig. 4A, panel b), as shown previously for the endogenous cut9 protein (47, 49). Significantly, the high-molecular-weight subpopulation of cut9p-HA peaked in fraction 6, along with lid1p-myc, at just greater than 19S. This high-molecular-weight fraction of cut9p-HA contained more slowly migrating forms of cut9p-HA which have been shown previously to be the result of phosphorylation (47).

Although the APC/C components cut9p and nuc2p have been shown to cosediment in wild-type cells, cut9p and nuc2p fail to cosediment at ~20S in lysates prepared from *cut9* or *nuc2* temperature-sensitive mutants (47). In order to determine whether temperature-sensitive mutations in *cut9* likewise might affect sedimentation of lid1p-myc, a *lid1::lid1-myc cut9-665* strain was constructed. Cells were grown to exponential phase at a permissive temperature, then shifted to a restrictive temperature (36°C) for 4 h. None of lid1p-myc sedimented at ~20S in the absence of *cut9*⁺ function; most sedimented in a smaller complex (Fig. 4A, panel c). The reciprocal experiment was also performed; cut9p-HA sedimentation in a *lid1-6* temperature-sensitive background was examined. Similar to lid1p-myc in the *cut9-665* mutant, most of the cut9-HA in the *lid1-6* mutant migrated with a lower sedimentation value (Fig. 4A, panel d). These data suggest that *lid1*⁺ function is required for the structural integrity of the APC/C.

lid1p-myc associates with cut9p-HA and nuc2p in vivo. The cosedimentation of lid1p-myc and cut9p-HA in sucrose gradients, as well as the failure of lid1p-myc to migrate in a high-molecular-weight complex in a *cut9-665* background, further suggested a physical interaction among lid1p and cut9p and/or other APC/C components. In order to determine directly whether lid1p-myc and cut9p-HA associate in vivo, native or denatured lysates were prepared from KGY1302 (*lid1::lid1-myc*), KGY1365 (*cut9::cut9-HA*), or KGY1366 (*lid1::lid1-myc cut9::cut9-HA*) cells. Lysates then were subjected to immunoprecipitation and immunoblot analysis with 9E10 or HA.11 antibodies. Probing with 9E10 revealed a band of ~100 kDa (lid1p-myc) in KGY1302 and KGY1366 native lysates, as well as in KGY1302 denatured lysates, but not in KGY1366 denatured lysates or KGY1365 native or denatured lysates. Conversely, probing with HA.11 revealed a band of ~90 kDa

FIG. 4. *lid1p-myc* is a component of the 20S APC/C. (A) *lid1p-myc* and *cut9p-HA* cosediment at ~20S. Fractions were collected from the bottom (fraction 1) of 10 to 30% sucrose gradients and then resolved by SDS-PAGE and immunoblotted with the 9E10 antibody to detect *lid1p-myc* (a and c) or with 12CA5 to detect *cut9p-HA* (b and d). Panels a and b show identical fractions from lysates prepared from the *lid1::lid1-myc cut9::cut9-3xHA* strain. Panel c shows fractions from the *lid1::lid1-myc cut9-665* strain that had been shifted to 36°C for 4 h prior to lysis. Panel d shows fractions from the *cut9::cut9-HA lid1-6* strain that had been shifted to 36°C for 4 h prior to lysis. Fraction numbers are indicated above the panels. Peak fractions of the 19S sedimentation marker thyroglobulin and the 11.3S sedimentation marker catalase are indicated below the panels. (B) *lid1p-myc* and *cut9p-HA* coimmunoprecipitate from KGY1366 (*lid1::lid1-myc cut9::cut9-HA*) whole-cell lysates but not from KGY1302 (*lid1::lid1-myc*) or KGY1365 (*cut9::cut9-HA*) cell lysates. KGY1302 (lanes 1, 2, 3, and 4), KGY1365 (lanes 5, 6, 7, and 8), or KGY1366 (lanes 9, 10, 11, and 12) native (N) (lanes 1, 3, 5, 7, 9, and 11) or denatured (D) (lanes 2, 4, 6, 8, 10, and 12) cell lysates were immunoprecipitated (IP) with 9E10 (lanes 1, 2, 5, 6, 9, and 10) or HA.11 (lanes 3, 4, 7, 8, 11, and 12) antibodies. Immunoprecipitates were resolved by SDS-PAGE and then subjected to immunoblotting with 9E10 (a) or HA.11 (b) antibodies. (C) *lid1p-myc* coimmunoprecipitates with *cut9p* and *nuc2p*. Polyclonal antibodies to *cut9p* (lanes 1 and 3) or *nuc2p* (lanes 2 and 4) were used for immunoprecipitation from wild-type (lanes 1 and 2) or KGY1302 (lanes 3 and 4) cell lysates. The immunoprecipitates were resolved by SDS-PAGE and blotted with 9E10 antibodies. *lid1p-myc* is indicated with an arrow.

(*cut9p-HA*) in KGY1365 and KGY1366 native cell lysates, as well as in KGY1365 denatured cell lysates, but not in KGY1366 denatured lysates or KGY1302 native or denatured lysates (Fig. 4B). Less *lid1p-myc* was immunoprecipitated with HA antibodies than with 9E10 antibodies, presumably because not all *lid1p* is within the 20S complex (compare Fig. 4A, panels a and b). Coimmunoprecipitation of *lid1p-myc* with known components of the *S. pombe* APC/C was confirmed by

using polyclonal antibodies against *nuc2p* and *cut9p* (a generous gift of M. Yanagida) (Fig. 4C).

***lid1p* is required for the multiubiquitination of *cut2p*.** To test directly whether *lid1p* was required for the E3 ligase activity of the APC/C, we examined the ubiquitination of *cut2p*, a known destruction box-containing APC/C target (12, 17), in the presence and absence of *lid1p* function. *cut2p* is required for sister chromatid separation and is targeted for APC/C-mediated destruction at the onset of anaphase (12). In order to detect *cut2p* readily, we placed sequences encoding 13 copies of the myc epitope tag at the 3' end of the *cut2* coding region; the tagged version was fully functional in that the *cut2::cut2-myc* strain was indistinguishable from the wild type in morphology and growth rate. A doublet at ~62 kDa corresponding to *cut2p-myc* was readily detected by immunoblotting with 9E10 (Fig. 5A); endogenous *cut2p* is 42 kDa and also migrates as a doublet in SDS-PAGE (12). The *cut2::cut2-myc* allele was crossed into an *mts3-1* strain with or without the *lid1-6* mutation. *mts3⁺* encodes subunit 14 of the 26S proteasome (15), and in the *mts3-1* mutant strain, multiubiquitinated conjugates accumulate at the nonpermissive temperature because of their failure to become degraded (4, 15). Thus, *cut2p-myc* would be expected to accumulate ubiquitin conjugates in the *mts3-1* mutant but only if the APC/C was active. To allow us to purify ubiquitin-conjugated proteins, a His₆-tagged version of ubiquitin was expressed in wild-type, *mts3-1*, *mts3-1 cut2::cut2-myc*, and *mts3-1 cut2::cut2-myc lid1-6* strains. Extracts from these strains were purified by using Ni²⁺-NTA resin and separated by SDS-PAGE, and ubiquitinated *cut2p-myc* was detected by immunoblotting. High-molecular-weight bands were detected in the *mts3-1 cut2::cut2-myc* strain expressing His₆-ubiquitin at the restrictive temperature (Fig. 5, lanes 4). These bands were much reduced in the *mts3-1 cut2::cut2-myc lid1-6* strain (Fig. 5, lanes 3). They were absent from the wild-type and *mts3-1* strains which do not express myc epitope-tagged proteins (Fig. 5, lanes 1 and 2). This result clearly establishes that *lid1p* is required for APC/C-mediated ubiquitination of *cut2p*.

***dim1⁺* function is required to maintain *lid1p* levels.** To investigate the basis for the negative genetic interaction between *lid1-6* and *dim1-35*, we examined whether an epitope-tagged version of *dim1p*, *dim1p-HA*, could be coimmunoprecipitated with *lid1p-myc*. We found no evidence for such a stable association (data not shown). We also examined the level of *lid1p-myc* in the *dim1-35* mutant. Interestingly, we found that it was reduced significantly (three- to fourfold in separate experiments as quantified with the use of a Molecular Dynamics Storm instrument) compared with that in wild-type cells grown at 36°C (Fig. 6A). In contrast, the level of *cut9p-HA* was not affected significantly in *dim1-35* cells (Fig. 6A and data not shown). To examine the effect of *dim1⁺* loss on *lid1p-myc* levels in a different manner and at a different arrest point within the cell cycle, we crossed the *lid1::lid1-myc* cassette into strain KGY1180, which contains a *dim1* null allele and a conditional expression cassette of the *dim1⁺* cDNA. More specifically, KGY1180 contains a single integrated version of *dim1⁺* cDNA under control of the thiamine-repressible *nmt1-T81* promoter. When grown in medium lacking thiamine, this strain is phenotypically wild type, but when placed in medium containing thiamine, the cell number stops increasing by 13 h and cells arrest in G₂ phase due to the loss of the essential *dim1p* (5). Protein lysates were prepared from samples of this strain collected periodically after a shift to thiamine-containing medium, and the level of *lid1p-myc* was determined by immunoblotting equal amounts of protein. As shown in Fig. 6B, *lid1p-myc* levels began to drop at 6 h and fell

FIG. 5. *lid1*⁺ and *dim1*⁺ functions are required for ubiquitination of cut2p. (A) Total cell lysates from wild-type (lane 1) or *cut2::cut2-myc* (lane 2) cells were resolved by SDS-PAGE and immunoblotted with 9E10. In the *cut2p-myc* doublet in lane 2, the lower-molecular mass protein is likely to be a degradation product of cut2p-myc. (B) His₆-ubiquitin was expressed from the *nmt1* promoter for 22 h at 25°C in wild-type cells (lane 1), *mts3-1* mutant cells (lane 2), *mts3-1 cut::cut2-myc lid1-6* cells (lane 3), or *mts3-1 cut::cut2-myc* cells (lane 4), and the strains were then shifted to 36°C for a further 4 h. His₆-ubiquitin conjugates were purified on Ni²⁺-NTA columns as described in Materials and Methods, and ubiquitinated cut2p-myc was detected by immunoblotting with 9E10. (C) As in panel B except that the strains examined were the wild-type (lane 1), *mts3-1* (lane 2), *mts3-1 cut::cut2-myc* (lanes 3 and 5), *mts3-1 cut::cut2-myc lid1-6* (lane 4), and *mts3-1 cut::cut2-myc dim1-35* (lane 6) strains. (D) Prior to purification on Ni²⁺-NTA columns, an aliquot of each lysate used for panel C was examined for the presence of cut2p-myc by immunoblotting with 9E10. Lysates were from wild-type cells (lane 1), *mts3-1* mutant cells (lane 2), *mts3-1 cut::cut2-myc* cells (lane 3), *mts3-1 cut::cut2-myc dim1-35* cells (lane 4), and *mts3-1 cut::cut2-myc lid1-6* cells (lane 5). The arrow indicates the position of cut2p-myc. Numbers on the left in panels A to C are molecular masses in kilodaltons.

to very low levels by 10 h (before complete cell cycle arrest), whereas the level of the loading control, arp3p, did not change. In a second experiment examining the consequence of the loss of *dim1*⁺ function for the abundance of lid1p-myc, we compared the level of lid1p-myc with that of cdc2p (Fig. 6C). Again, lid1p-myc levels were significantly reduced by 10 h after shutoff of *nmt1-T81 dim1*⁺, whereas the level of cdc2p did not change. Thus, the abundance of lid1p-myc depends upon the function of *dim1*⁺.

To determine whether the loss of lid1p in *dim1-35* cells affected the abundance of the 20S APC/C, we compared the sedimentation behaviors of cut9p-HA from wild-type and *dim1-35* mutant cells grown at 36°C for 4 or 6 h. When isolated from *dim1-35* cells, cut9p-HA sedimented more broadly in the gradient, with the largest concentrations detected in fractions 12 and 13 rather than 7 and 8 (compare Fig. 7A with B and C with D). Fractions 7 and 8 correspond to the position of the 20S complex. Indeed, the majority of cut9p-HA sedimented more slowly than that isolated from wild-type cells, indicating that it was coming out of, or not being as efficiently incorporated into, the 20S APC/C complex. Further, the cut9p-HA that was present at 20S was not phosphorylated to the same

extent as in wild-type cells. The blot in Fig. 7D was overexposed relative to that in Fig. 7C to illustrate this point. We conclude from these data that *dim1*⁺ function affects the abundance and posttranslational modification of the APC/C in *S. pombe*.

***dim1*⁺ function is required for the multiubiquitination of cut2p.** Given that the abundance of the 20S APC/C complex declined in the absence of *dim1*⁺ function, we reasoned that the activity of the APC/C might also be compromised. To test this possibility, we constructed a *dim1-35 mts3-1 cut2::cut2-myc* strain and introduced the His₆-tagged version of ubiquitin into it. When this strain was tested for the ubiquitination of cut2p-myc following a shift to the nonpermissive temperature, in parallel with the strains described above, we found that cut2p-myc ubiquitination was abolished in the absence of *dim1*⁺ function (Fig. 5C, lane 6). The immunoblot in Fig. 5D shows that cut2p-myc was present in the *dim1-35* and *lid1-6* protein lysates from which the ubiquitin-conjugated proteins were purified. Taken together, these data indicate that *dim1*⁺ function is required for APC/C function.

lid1p-myc protein levels are constant through the cell cycle. Because of the decrease in lid1p levels in the absence of *dim1*

FIG. 6. lid1p-myc levels drop in the absence of *dim1*⁺ function. (A) Wild-type (wt) or *dim1-35* (*dim1*) cells producing lid1p-myc or cut9p-HA were grown to mid-log phase at 25°C and then shifted to 36°C for 4 h. Total protein lysates were prepared and immunoblotted with 9E10 (left panel) or 12CA5 (right panel) antibodies. Immunoblotting with anti-arp3p antibodies provided a loading control. (B and C) The *dim1::his3*⁺ strain carrying a single integrated copy of *nmt1-T81::dim1*⁺ (KGY1180) and the *lid1::lid1-myc* cassette was grown in minimal medium lacking thiamine and then shifted to rich medium containing thiamine to repress expression of the *nmt1-T81::dim1*⁺ cassette. Samples were collected for analysis at the indicated times (given in hours). Protein lysates were prepared and immunoblotted for lid1p-myc (top panels), arp3p (bottom panel in panel B), or cdc2p with anti-PSTAIR monoclonal antibody (bottom panel in panel C).

FIG. 7. *dim1*⁺ function affects the abundance of the 20S APC/C. *cut9::cut9-HA* (A and C) and *cut9::cut9-HA dim1-35* (B and D) cells were grown to mid-log phase at 25°C and shifted to 36°C for 4 h (A and B) or 6 h (C and D). Cell lysates were prepared and layered over sucrose gradients. Fractions were collected from the bottom (fraction 1), resolved by SDS-PAGE, and immunoblotted with 12CA5 monoclonal antibodies. Reactive proteins were visualized by ECL. Fraction numbers are indicated above the panels. Peak fractions of the 19S sedimentation marker thyroglobulin and the 11.3S sedimentation marker catalase are indicated below the panels.

function in both *dim1-35* cells, which arrest in mitosis, and *dim1* null cells, which arrest at the G₂/M transition, we became interested to determine whether lid1p-myc levels varied through the cell cycle. To this end, a synchronized G₂ population of *lid1::lid1-myc cut9::cut9-HA* (KGY1366) cells was isolated by centrifugal elutriation. Samples were collected as cells progressed through two synchronous cell cycles. The levels of cut9p-HA and cdc13p (as positive controls for cell cycle periodicity), arp3p (as a loading control), and lid1p-myc were determined by immunoblotting. The hyperphosphorylated form of cut9p-HA was observed just prior to each peak of septation (Fig. 8a, *t* = 40 min and *t* = 180 min), as was reported previously (47). cdc13p levels are known to fall during mitosis (7, 13), and indeed we observed that cdc13p levels dropped coincident with each septation peak (Fig. 8b, *t* = 60 min and *t* = 200 min). In contrast, lid1p-myc levels did not undergo any significant changes through the cell cycle, nor did lid1p-myc undergo any detectable shifts in electrophoretic mobility suggestive of posttranslational modifications (Fig. 8c). We also examined lid1p-myc levels in a *cdc25-22* block-and-release experiment, which provides a higher degree of cell cycle synchrony. Again, we did not observe any fluctuation in lid1p-myc levels (data not shown).

DISCUSSION

Identification of lid1p, a component of the *S. pombe* APC/C. dim1p is a 17-kDa, evolutionarily conserved, essential protein. A temperature-sensitive allele of *dim1*, *dim1-35*, causes arrest in mitosis and inability to initiate chromosome segregation, although spindle elongation occurs. In contrast, cells containing a null mutation in *dim1* are unable to enter mitosis and block at the G₂/M transition (5). To gain further insight into dim1p function, we performed a synthetic lethal screen aimed at identifying proteins that interact with dim1p. Here, we report our characterization of one *lid* (for lethal in *dim1-35*) mutant, the *lid1-6* mutant. In a *dim1*⁺ background, *lid1-6* causes a temperature-sensitive phenotype. Specifically, *lid1-6* mutant cells are unable to undergo anaphase at the restrictive temperature; short metaphase spindles form but do not elongate, and the chromosomes do not separate. Based on this

phenotype, which is similar to that of *cut4* and *cut9* mutants (40, 49), we hypothesized that *lid1*⁺ might encode a component of the *S. pombe* APC/C, since cut4p and cut9p are established APC/C components (47, 49). The APC/C is a 20S complex that acts as an E3 ubiquitin ligase essential for anaphase onset in all eukaryotes examined (reviewed in references 8, 17, 26, and 44).

In order to test the hypothesis that *lid1* may encode an APC/C component, we cloned the *lid1* gene by complementation of the *lid1-6* temperature-sensitive phenotype. DNA se-

FIG. 8. lid1p-myc levels remain constant through the cell cycle. KGY1366 cells were synchronized in early G₂ by centrifugal elutriation and then released into fresh medium. As cells progressed through the cell cycle, synchrony was monitored by determination of septation index at 20-min intervals (e). At the same intervals, samples were collected for analysis of cut9p-HA (a), cdc13p (b), lid1p-myc (c), and arp3p (d) protein levels.

```

293 --GKDTT--SVQDFMHLMLGKASAEQLTLLMQLTVKGLKGLQSSIESYSSQKLVIHSLQSGSESL 360
304 --YSDTFPSLQLELYQFMNGIPSDAKLEWINERVDVLKNWERAMVNSYSLIFCQEFVIPACERLT 372
273 --GYGKLKLS---ELEALFLGEISCDLELWCLNSVGEKNFKRWKYLGEAYQKTVQILTLFVPACERIT 338

361 YHLSSELKGM--ASWKQKYEPLGLDAAIGEEAITAVGSPILKANELLQVIDSSMKNFKAFFRWLYVAMLR-- 427
373 VLLSSARGK--SIWGHMKQNTLDAKLVEDCLATLQYLNQVNSFLNCLFEKKYMKHPISWLYAIV--- 438
439 IYVEKLRAILQAFSIQNKLSYTSDLTAVEVLLKSSQKLLTMTLNSIIGLGRDETLEKFFIWFENDRLH--- 406

```

FIG. 9. Sequence comparison of lid1p with human APC4 and *S. cerevisiae* Apc4p. The indicated amino acid sequences of human APC4 (top line), *S. pombe* lid1p (middle line), and *S. cerevisiae* Apc4p (bottom line) were aligned. Amino acids identical in lid1p and APC4 or Apc4p are indicated by vertical dashes. Amino acids identical in APC4 and Apc4p are indicated by asterisks.

quencing and hypothetical translation of the *lid1* gene provided no clues as to the function of *lid1*. However, consistent with the notion that *lid1*⁺ encodes a component of the APC/C, an epitope-tagged variant of lid1p, lid1p-myc, coimmunoprecipitated with several other proteins, including cut9p and nuc2p, which are known members of the *S. pombe* APC/C. The results of sucrose gradient analysis also are consistent with the interpretation that lid1p is a component of the *S. pombe* APC/C; lid1p-myc cosediments with cut9p-HA at ~20S, the sedimentation profile of lid1p-myc depends upon functional *cut9*⁺, and the ability of cut9p-HA to sediment at 20S depends upon functional *lid1*⁺. Furthermore, lid1p function is required for the multiubiquitination of cut2p, a known target of the APC/C (12, 13). These data establish that lid1p is a component of the *S. pombe* APC/C and also that its function is necessary for maintaining the integrity of the 20S APC/C.

Our comparison of the predicted lid1 protein sequence with sequences available in the databases revealed no significant homologies or motifs. Our failure to detect a budding yeast homolog was especially surprising given our assumption that the APC/C components would be conserved between budding and fission yeasts. However, while this work was in progress, the sequences of most, if not all, components of the human and yeast APC/Cs were reported (50, 52). When the sequence of the human APC4 protein was used as a query to probe databases, limited similarity between it and *S. pombe* ORF Z97209 was observed, but no homolog was detected in budding yeast (50). *S. pombe* ORF Z97209 encodes lid1p. Budding yeast Apc4p also was reported to have weak similarity with *S. pombe* ORF Z97209 (52). We have aligned lid1p with APC4 and Apc4p and have found a central region of 133 or 134 amino acids which contains significant sequence conservation (Fig. 9). There is 24% identity and 34% similarity between *S. pombe* lid1p and human APC4 in this region and 22% identity and 31% similarity between *S. pombe* lid1p and *S. cerevisiae* Apc4p. Outside this region, there is little sequence similarity among the three proteins, although there are two other stretches of sequence similarity between *S. pombe* lid1p and human APC4 (data not shown). Taken together, these protein sequence comparisons indicate that lid1p, APC4, and Apc4p are distantly related, and it will be interesting to determine whether they are conserved functionally (50). The isolation of a conditional lethal mutation in the *S. pombe* component most similar to both human APC4 and budding yeast Apc4p provides an excellent opportunity to test this directly; the lack of sequence conservation makes it unlikely that cross complementation of the yeast null alleles would be successful.

dim1p is required for APC/C integrity and activity. The APC/C is active towards destruction box-containing substrates only during mitosis and G₁ phase (reviewed in references 8, 17, 26, and 44). Its activation towards mitotic cyclins *in vitro* has been attributed to phosphorylation of its components rather

than to alterations in its composition or abundance (reviewed in references 8, 17, 26, and 44). For these reasons, we were surprised to learn that the abundance of lid1p fell in the absence of *dim1*⁺ function. However, this observation provides a reasonable explanation for the synthetic lethal interaction between *lid1-6* and *dim1-35*. In a *dim1-35* mutant background, there is probably an insufficient amount of functional lid1-6p to support cell cycle progression. The loss of lid1p in the absence of *dim1* activity is not due to a specific cell cycle block, because lid1p was lost both in *dim1-35* and in *dim1* null cells. *dim1-35* cells arrest in mitosis with a cut-like phenotype, but *dim1* null cells arrest at the G₂/M transition (5). In terms of understanding the biochemical mechanism of dim1p action, we can now direct our efforts to understanding the role of dim1p in maintaining the level of lid1p. dim1p is not stably associated with lid1p (our unpublished observations), and it will be very instructive to learn whether dim1p affects the stability or synthesis of lid1p or *lid1* mRNA. The interaction with lid1p is probably insufficient to explain the essential role of dim1p, however, since overexpression of *lid1*⁺ does not rescue the *dim1-35* mutant (data not shown).

By determining the sedimentation behavior of cut9p-HA in *dim1-35* cells, we have found that the reduction of lid1p in *dim1* mutants is translated into a lower level of the 20S APC/C complex. This is not surprising given the interdependence of various components for the integrity of the complex. As two examples, in a *cut9* mutant, neither nuc2p nor lid1p remains in a 20S complex; in a *nuc2* or *lid1* mutant, cut9p does not sediment at 20S (reference 47 and this study). Thus, the failure of *dim1-35* cells to execute chromosome segregation can be most easily explained by a reduction of APC/C activity caused by reduced levels of lid1p and the consequent reduction in the amount of the APC/C complex. Consistent with this interpretation, we have shown that loss of *dim1*⁺ function abrogates the multiubiquitination of cut2p-myc. Whether the failure of cells carrying the *dim1* null allele to enter mitosis can be attributed to the lack of APC/C activity remains to be determined. In addition to the reduced levels of the 20S complex in the *dim1-35* mutant, we noted a reduction in the phosphorylation of cut9p-HA. Whether this is a consequence or a cause of the reduction in 20S complex abundance or an unrelated phenomenon also remains to be determined.

In summary, we have uncovered a dependency between *dim1*⁺ function and the function of the APC/C. Future efforts will be directed at understanding how dim1p affects lid1p abundance and whether it acts through intermediate components to do so.

ACKNOWLEDGMENTS

We thank J. Bahler, J. Pringle, and K. Nasmyth for providing the epitope tagging cassettes, K. Gull for the TAT-1 monoclonal antibody, S. Moreno for tagged ubiquitin construct and advice on its use, and M. Yanagida for polyclonal antibodies to cut9p and nuc2p. We are grateful to Hayes McDonald for advice on epitope tagging, Ryoma Ohi and Jennifer Morrell for constructive comments on the manuscript, and all members of the Gould lab for their support and advice during the course of this work.

This work was supported by NIH grant 47728 to K.L.G. L.D.B. was supported by a National Science Foundation Graduate Research Fellowship. K.L.G. is an associate investigator of the Howard Hughes Medical Institute.

REFERENCES

- Bahler, J., J. Q. Wu, M. S. Longtine, N. G. Shah, A. McKenzie III, A. B. Steever, A. Wach, P. Philippsen, and J. R. Pringle. 1998. Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. *Yeast* 14:943-951.

2. Balasubramanian, M. K., D. McCollum, and K. L. Gould. 1997. Cytokinesis in fission yeast *Schizosaccharomyces pombe*. *Methods Enzymol.* **283**:494–506.
3. Barbet, N., W. J. Muriel, and A. M. Carr. 1992. Versatile shuttle vectors and genomic libraries for use with *Schizosaccharomyces pombe*. *Gene* **114**:59–66.
4. Benito, J., C. Martin-Castellanos, and S. Moreno. 1998. Regulation of the G1 phase of the cell cycle by periodic stabilization and degradation of the p25rum1 CDK inhibitor. *EMBO J.* **17**:482–497.
5. Berry, L. D., and K. L. Gould. 1997. Fission yeast dim1(+) encodes a functionally conserved polypeptide essential for mitosis. *J. Cell Biol.* **137**:1337–1354.
6. Charles, J. F., S. L. Jaspersen, R. L. Tinker-Kulberg, L. Hwang, A. Szidon, and D. O. Morgan. 1998. The Polo-related kinase Cdc5 activates and is destroyed by the mitotic cyclin destruction machinery in *S. cerevisiae*. *Curr. Biol.* **8**:497–507.
7. Ciechanover, A. 1994. The ubiquitin-proteasome proteolytic pathway. *Cell* **79**:13–21.
8. Cohen-Fix, O., and D. Koshland. 1997. The metaphase-to-anaphase transition: avoiding a mid-life crisis. *Curr. Opin. Cell Biol.* **9**:800–806.
9. Creanor, J., and J. M. Mitchison. 1996. The kinetics of the B cyclin p56cdc13 and the phosphatase p80cdc25 during the cell cycle of the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* **109**:1647–1653.
10. Evans, T., E. T. Rosenthal, J. Youngbloom, D. Distel, and T. Hunt. 1983. Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell* **33**:389–396.
11. Fikes, J. D., D. M. Becker, F. Winston, and L. Guarente. 1990. Striking conservation of TFIID in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. *Nature* **346**:291–294.
12. Funabiki, H., K. Kumada, and M. Yanagida. 1996. Fission yeast Cut1 and Cut2 are essential for sister chromatid separation, concentrate along the metaphase spindle and form large complexes. *EMBO J.* **15**:6617–6628.
13. Funabiki, H., H. Yamano, K. Nagao, H. Tanaka, H. Yasuda, T. Hunt, and M. Yanagida. 1997. Fission yeast Cut2 required for anaphase has two destruction boxes. *EMBO J.* **16**:5977–5987.
14. Glotzer, M., A. W. Murray, and M. W. Kirschner. 1991. Cyclin is degraded by the ubiquitin pathway. *Nature* **349**:132–138.
15. Gordon, C., G. McGurk, M. Wallace, and N. D. Hastie. 1996. A conditional lethal mutant in the fission yeast 26 S protease subunit mts3+ is defective in metaphase to anaphase transition. *J. Biol. Chem.* **271**:5704–5711.
16. Gould, K. L., S. Moreno, D. J. Owen, S. Sazer, and P. Nurse. 1991. Phosphorylation at Thr167 is required for *Schizosaccharomyces pombe* p34cdc2 function. *EMBO J.* **10**:3297–3309.
17. Hershko, A. 1997. Roles of ubiquitin-mediated proteolysis in cell cycle control. *Curr. Opin. Cell Biol.* **9**:788–799.
18. Hirano, T., Y. Hiraoka, and M. Yanagida. 1988. A temperature-sensitive mutation of the *Schizosaccharomyces pombe* gene nuc2+ that encodes a nuclear scaffold-like protein blocks spindle elongation in mitotic anaphase. *J. Cell Biol.* **106**:1171–1183.
19. Hochstrasser, M. 1995. Ubiquitin, proteasomes, and the regulation of intracellular protein degradation. *Curr. Opin. Cell Biol.* **7**:215–223.
20. Hoffman, C. S. 1993. Preparation of yeast DNA, p. 13.11.1–13.11.4. In F. A. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), *Current protocols in molecular biology*. John Wiley and Sons, New York, N.Y.
21. Hoheisel, J. D., E. Maier, R. Mott, L. McCarthy, A. V. Grigoriev, L. C. Schalkwyk, D. Nizetic, F. Frances, and H. Lehrach. 1993. High-resolution cosmid and P1 maps spanning the 14 Mbp genome of the fission yeast *Schizosaccharomyces pombe*. *Cell* **73**:109–120.
22. Irniger, S., and K. Nasmyth. 1997. The anaphase-promoting complex is required in G1 arrested yeast cells to inhibit B-type cyclin accumulation and to prevent uncontrolled entry into S-phase. *J. Cell Sci.* **110**:1523–1531.
23. Irniger, S., S. Piatti, C. Michaelis, and K. Nasmyth. 1995. Genes involved in sister chromatid separation are needed for B-type cyclin proteolysis in budding yeast. *Cell* **81**:269–278.
24. Juang, Y. L., J. Huang, J. M. Peters, M. E. McLaughlin, C. Y. Tai, and D. Pellman. 1997. APC-mediated proteolysis of Ase1 and the morphogenesis of the mitotic spindle. *Science* **275**:1311–1314.
25. Keeney, J. B., and J. D. Boeke. 1994. Efficient targeted integration at leu1-32 and ura4-294 in *Schizosaccharomyces pombe*. *Genetics* **136**:849–856.
26. King, R. W., R. J. Deshaies, J. M. Peters, and M. W. Kirschner. 1996. How proteolysis drives the cell cycle. *Science* **274**:1652–1659.
27. King, R. W., J. M. Peters, S. Tugendreich, M. Rolfe, P. Hieter, and M. W. Kirschner. 1995. A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. *Cell* **81**:279–288.
28. Kumada, K., S. Su, M. Yanagida, and T. Toda. 1995. Fission yeast TPR-family protein nuc2 is required for G1-arrest upon nitrogen starvation and is an inhibitor of septum formation. *J. Cell Sci.* **108**:895–905.
29. Lamb, J. R., W. A. Michaud, R. S. Sikorski, and P. A. Hieter. 1994. Cdc16p, Cdc23p and Cdc27p form a complex essential for mitosis. *EMBO J.* **13**:4321–4328.
30. Lees, E. 1995. Cyclin dependent kinase regulation. *Curr. Opin. Cell Biol.* **7**:773–780.
31. McCollum, D., A. Feoktistova, M. Morphew, M. Balasubramanian, and K. L. Gould. 1996. The *Schizosaccharomyces pombe* actin-related protein, Arp3, is a component of the cortical actin cytoskeleton and interacts with profilin. *EMBO J.* **15**:6438–6446.
32. Michaelis, C., R. Ciosk, and K. Nasmyth. 1997. Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. *Cell* **91**:35–45.
33. Moreno, S., A. Klar, and P. Nurse. 1991. Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. *Methods Enzymol.* **194**:795–823.
34. Morgan, D. O. 1995. Principles of CDK regulation. *Nature* **374**:131–134.
35. Murray, A. W., M. J. Solomon, and M. W. Kirschner. 1989. The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. *Nature* **339**:280–286.
36. Peters, J. M., R. W. King, C. Hoog, and M. W. Kirschner. 1996. Identification of BIME as a subunit of the anaphase-promoting complex. *Science* **274**:1199–1201.
37. Pines, J. 1995. Cyclins and cyclin-dependent kinases: a biochemical view. *Biochem. J.* **308**:697–711.
38. Prentice, H. L. 1992. High efficiency transformation of *Schizosaccharomyces pombe* by electroporation. *Nucleic Acids Res.* **20**:621.
39. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
40. Samejima, I., and M. Yanagida. 1994. Bypassing anaphase by fission yeast cut9 mutation: requirement of cut9+ to initiate anaphase. *J. Cell Biol.* **127**:1655–1670.
41. Sazer, S., and S. W. Sherwood. 1990. Mitochondrial growth and DNA synthesis occur in the absence of nuclear DNA replication in fission yeast. *J. Cell Sci.* **97**:509–516.
42. Schwab, M., A. S. Lutum, and W. Seufert. 1997. Yeast Hct1 is a regulator of Clb2 cyclin proteolysis. *Cell* **90**:683–693.
43. Shirayama, M., W. Zachariae, R. Ciosk, and K. Nasmyth. 1998. The Polo-like kinase Cdc5p and the WD-repeat protein Cdc20p/fizzy are regulators and substrates of the anaphase promoting complex in *Saccharomyces cerevisiae*. *EMBO J.* **17**:1336–1349.
44. Townsley, F. M., and J. V. Ruderman. 1998. Proteolytic ratchets that control progression through mitosis. *Trends Cell Biol.* **8**:238–244.
45. Visintin, R., S. Prinz, and A. Amon. 1997. CDC20 and CDH1: a family of substrate-specific activators of APC-dependent proteolysis. *Science* **278**:460–463.
46. Woods, A., T. Sherwin, R. Sasse, T. H. MacRae, A. J. Baines, and K. Gull. 1989. Definition of individual components within the cytoskeleton of *Trypanosoma brucei* by a library of monoclonal antibodies. *J. Cell Sci.* **93**:491–500.
47. Yamada, H., K. Kumada, and M. Yanagida. 1997. Distinct subunit functions and cell cycle regulated phosphorylation of 20S APC/cyclosome required for anaphase in fission yeast. *J. Cell Sci.* **110**:1793–1804.
48. Yamamoto, A., V. Guacci, and D. Koshland. 1996. Pds1p, an inhibitor of anaphase in budding yeast, plays a critical role in the APC and checkpoint pathway(s). *J. Cell Biol.* **133**:99–110.
49. Yamashita, Y. M., Y. Nakaseko, I. Samejima, K. Kumada, H. Yamada, D. Michaelson, and M. Yanagida. 1996. 20S cyclosome complex formation and proteolytic activity inhibited by the cAMP/PKA pathway. *Nature* **384**:276–279.
50. Yu, H., J. M. Peters, R. W. King, A. M. Page, P. Hieter, and M. W. Kirschner. 1998. Identification of a cullin homology region in a subunit of the anaphase-promoting complex. *Science* **279**:1219–1222.
51. Zachariae, W., and K. Nasmyth. 1996. TPR proteins required for anaphase progression mediate ubiquitination of mitotic B-type cyclins in yeast. *Mol. Biol. Cell* **7**:791–801.
52. Zachariae, W., A. Shevchenko, P. D. Andrews, R. Ciosk, M. Galova, M. J. Stark, M. Mann, and K. Nasmyth. 1998. Mass spectrometric analysis of the anaphase-promoting complex from yeast: identification of a subunit related to cullins. *Science* **279**:1216–1219.
53. Zachariae, W., T. H. Shin, M. Galova, B. Obermaier, and K. Nasmyth. 1996. Identification of subunits of the anaphase-promoting complex of *Saccharomyces cerevisiae*. *Science* **274**:1201–1204.