Role of Hcn1 and Its Phosphorylation in Fission Yeast Anaphase-promoting Complex/Cyclosome Function*

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The anaphase-promoting complex/cyclosome (APC/C)4 is a conserved multisubunit ubiquitin ligase required for the degradation of key cell cycle regulators. The APC/C becomes active at the metaphase/anaphase transition and remains active during G1 phase. One mechanism linked to activation of the APC/C is phosphorylation. Although many sites of mitotic phosphorylation have been identified in core components of the APC/C, the consequence of any individual phosphorylation event has not been elucidated in vivo. In this study, we show that Hcn1 is an essential core component of the fission yeast APC/C and is critical for maintaining complex integrity. Moreover, Hcn1 is a phosphoprotein in vivo. Phosphorylation of Hcn1 occurs at a single Cdk1 site in vitro and in vivo. Mutation of this site to alanine, but not aspartic acid, compromises APC/C function and leads to a specific defect in the completion of cell division.

The anaphase-promoting complex/cyclosome (APC/C)4 is a multiprotein ubiquitin-protein isopeptide ligase that was first identified based on its role in facilitating the ubiquitination of A- and B-type cyclins, thereby targeting them for proteasome-mediated destruction during mitosis (1–3). Although mitotic phase cyclins were the first targets known, many other APC targets have been identified subsequently (4), including securin, the destruction of which is required for chromosome segregation (5–7). Substrates are recognized by the APC/C based on the presence of short consensus motifs within them, such as the D-box, the KEN motif, and the A-box (8).

The majority of APC/C subunits (at least 13 in yeast) are stably associated throughout the cell cycle in an inactive core complex. The addition of transiently expressed CDC20 protein family members and phosphorylation events contribute to APC/C activation during mitosis and G1 phase. Although active APC/C preparations treated with phosphatase lose activity (9–11), it remains unclear which phosphorylation event(s) on which subunit(s) are responsible for this observation. It is also possible that no individual phosphorylation event alone has much effect, but rather it is the combination of many phosphorylation events that serves to alter complex activity. In this regard, it is clear that both CDC20 and core APC/C components are phosphorylated during mitosis. Recently, roles of CDC20 phosphorylation in both positive and negative APC/C regulation have emerged (12–16), suggesting that CDC20 phosphorylation might be a common target of cell signaling pathways that affect mitotic progression by altering the timing of APC/C activity (17).

Several components of the core APC/C are phosphoproteins (18–22), and in vitro, the purified mitotic protein kinases Cdk1 and Plk (Polo in Drosophila, Cdc5 in Saccharomyces cerevisiae, and Plo1 in Schizosaccharomyces pombe) phosphorylate multiple subunits (1). To begin tackling the contribution of Cdk1 phosphorylation to APC/C function, all consensus Cdk1 phosphorylation sites in Cdc27 (APC3), Cdc16 (APC6), and Cdc23 (APC8) were altered to alanines (21). There were some defects in mitotic exit and CDC20 binding and increased sensitivity to the spindle checkpoint in cells expressing the APC/C complex missing these serine and threonine residues (21). However, the lack of significant defects in the context of previous data indicating an essential role of APC/C phosphorylation argues that critical phosphorylation events were not identified. Indeed, phosphorylation of other subunits in vitro and residual phosphorylation of the subunits targeted for mitogenesis in vivo were detected. A recent investigation of human APC/C phosphorylation by mass spectrometry was more comprehensive in terms of site identification and reported that there are >43 sites of phosphorylation within the human APC/C, 34 of which are specific to mitosis (23). This study also demonstrated that Cdk1 is able to phosphorylate many of the identified sites on more than three subunits; Plk is able to phosphorylate others, and still other sites are not generated by either of these protein kinases in vitro, suggesting the involvement of additional protein kinases in APC/C regulation. Despite the wealth of data from this study on APC/C phosphorylation sites, the picture is still incomplete, as three APC/C subunits were not identified in the mass spectrometric analysis (23). In terms of the role of these phosphorylation events in APC/C regulation, Cdk1 and Plk phosphorylation increases APC/C activity in vitro in a CDC20-dependent manner (21, 23–25). However, the roles of particu-
lar phosphorylation events in particular core subunits have not been determined. Interestingly, the timing of phosphorylation events in different subunits was found to vary during mitosis, raising the possibility that distinct phosphorylation events might have different functional consequences (23).

We purified the S. pombe APC/C previously using tandem affinity purification and identified a total of 13 core components, including Hcn1, by mass spectrometry (26). Hcn1 had been identified previously as a high copy suppressor of a cut9-665 mutant (27). It was assumed to be an APC/C component because of its homology to the S. cerevisiae APC/C component (Cdc26) and because Cdc26 can also suppress cut9 (27). However, it had not been shown previously to co-purify with bona fide APC/C components. Here, we have extended our analysis of the role of Hcn1 within the S. pombe APC/C and addressed the role of Hcn1 phosphorylation in cell cycle progression. We found that Hcn1, unlike S. cerevisiae Cdc26, is an essential core APC/C component. It binds Cut9 directly and is required to link different APC/C subcomplexes together. We have mapped the single site of phosphorylation within Hcn1 to Ser48, a consensus site for Cdk1. Mutational analysis indicated that Hcn1 phosphorylation is not an essential modification, but mutation of Ser48 to alanine induced a novel S. pombe APC/C phenotype. hcn1-S48A cells displayed a pronounced delay in the final stages of cell division because of a reduction in the amount of the Ace2 transcription factor.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Molecular Biology Methods**—The S. pombe strains used in this study (Table 1) were grown in yeast extract medium or Edinburgh minimal medium with the appropriate supplements (28). Expression of constructs under the control of the thiamine-repressible nmt promoter system was performed as described previously (29). Standard genetic and recombinant DNA methods were used except where noted. Gene fragments were obtained by PCR amplification from S. pombe genomic DNA. Yeast transformations were performed using either a lithium acetate method (30) or electroporation (31). Cells were labeled with [32P]orthophosphate as detailed previously (32).

**Cell Cycle Synchronization**—To obtain synchronous cultures of cells, small cells in early G2 phase were isolated from 4-liter cultures that had been grown in yeast extract medium at 25 °C to mid-log phase by centrifugal elutriation using a Beckman JE-5.0 rotor. The isolated small cells were filtered immediately and resuspended in yeast extract medium at the indicated temperatures and recombinant DNA methods were used except where noted. Standard genetic and recombinant DNA methods were used except where noted. Gene fragments were obtained by PCR amplification from S. pombe genomic DNA. Yeast transformations were performed using either a lithium acetate method (30) or electroporation (31). Cells were labeled with [32P]orthophosphate as detailed previously (32).

**In Vivo Tagging and Gene Deletion**—Strains expressing epitope-tagged versions of wild-type and mutant Hcn1 were constructed using a PCR-based approach as described previously (33). hcn1+ was tagged at its endogenous locus at its 3‘-end with a variety of tag-Kan cassette, including enhanced green fluorescent protein and Myc13. Appropriate tagging was confirmed by PCR and immunoblot inspection as appropriate. All tagged strains were viable at temperatures ranging from 25 to 36 °C. The hcn1+ coding sequences were replaced with the ura4+ gene using a one-step PCR-based approach as described previously (33). The appropriate deletion was confirmed by

**TABLE 1**

Strains used in this study

| Strain    | Genotype                                      |
|-----------|-----------------------------------------------|
| KGY33     | h′ cut23-Myc13::kan′ ade6-M210 leu1-32 ura4-D18 |
| KGY2836   | h′ ade6-M210 leu1-32 ura4-D18                 |
| KGY1336   | h′ lid1-Myc13::kan′ ade6-M210 leu1-32 ura4-D18 |
| KGY1551   | h′ hcn1-GFP::nuc2-663                         |
| KGY1552   | h′ hcn1-S48A-GFP::kan′ nuc2-663               |
| KGY1565   | h′ ace2-Myc13::kan′ hcn1-GFP::kan′ ade6-M210 leu1-32 ura4-D18 |
| KGY1566   | h′ ace2-Myc13::kan′ hcn1-S48A-GFP::kan′ ade6-M210 leu1-32 ura4-D18 |
| KGY1630   | h′ hcn1-S48A ade6-M210 leu1-32 ura4-D18       |
| KGY1833   | h′ hcn1-S48D ade6-M210 leu1-32 ura4-D18       |
| KGY1834   | h′ hcn1-S48A-GFP::kan′ mts3-1                 |
| KGY1886   | h′ hcn1-GFP::kan′ ade6-M210X leu1-32 ura4-D18 |
| KGY2026   | h′ hcn1-Myc13::kan′ lid1-TAP::kan′ ade6-M210 leu1-32 ura4-D18 |
| KGY2057   | h′ cut9-HA::kan′ lid1-TAP::kan′ ade6-M210 leu1-32 ura4-D18 |
| KGY2229   | h′ hcn1-S48A-GFP::kan′ lid1-6                 |
| KGY2231   | h′ hcn1-GFP::kan′ lid1-6                      |
| KGY2306   | h′ hcn1-Myc13::kan′ mts3-1 ade6-M210 leu1-32 |
| KGY2830   | h′ mts3-1 leu1-32                            |
| KGY3299   | h′ hcn1-Myc13::kan′ ade6-M210 leu1-32 ura4-D18 |
| KGY3583   | h′ hcn1-Myc::kan′ cut9-HA::kan′ ade6-M210 ura4-D18 |
| KGY3585   | h′ hcn1-Myc::kan′ cut9-HA::kan′ ade6-M210 ura4-D18 |
| KGY3590   | h′ lid1-TAP::kan′ ade6-M210 leu1-32 ura4-D18  |
| KGY3656   | h′ hcn1-Myc13::kan′ ade6-M210 leu1-32 ura4-D18 |
| KGY3668   | h′ hcn1-Myc13::kan′ ade6-M210 leu1-32 ura4-D18 |
| KGY3732   | h′ cut9-HA::kan′ ade6-M210X leu1-32 ura4-D18  |
| KGY4015   | h′ hcn1-Myc13::kan′ cut9-HA::kan′ cut9-HA::kan′ |
| KGY4578   | h′ hcn1-Myc13::kan′ cut9-HA::kan′ cut9-HA::kan′ |
| KGY4881   | h′ hcn1-Myc13::kan′ cut9-HA::kan′ cut9-HA::kan′ |
| KGY4775   | h′ hcn1-S48A-GFP::kan′ ade6-M210X leu1-32 ura4-D18 |
| KGY5632   | h′ hcn1-GFP::kan′ mts3-1                      |
| KGY6080   | h′ hcn1-GFP::kan′ cut9-HA::kan′ ade6-M210 leu1-32 ura4-D18 |
| KGY6158   | h′ lid1-Myc13::kan′ hcn1-S48A-GFP::kan′ ade6-M210 leu1-32 ura4-D18 |
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PCR amplification using primers inside the \( ura4^+ \) gene and primers outside the disruption cassette.

**Immunoprecipitation and Immunoblotting**—Whole cell lysates were prepared in Nonidet P-40 buffer, followed by anti-hemagglutinin (HA), anti-green fluorescent protein (GFP), or anti-Myc immunoprecipitations as described previously (32, 34). Denatured lysates were prepared as described previously (35). Protein samples were resolved on 10% SDS-polyacrylamide gels and subsequently transferred to 0.2-μm nitrocellulose membrane (Bio-Rad) or Immobilon (Millipore). Immunoblotting was done with anti-HA (12CA5; 0.3 μg/ml), anti-Myc (9E10; 1.1 μg/ml), anti-PSTAIRE peptide (1:5000; Sigma), or anti-GFP (0.2 μg/ml; Roche Applied Science) monoclonal antibody. The primary antibodies were detected with horseradish peroxidase-conjugated goat anti-IgG secondary antibodies (0.4 mg/ml; Jackson ImmunoResearch Laboratories, Inc.) at a dilution of 1:50,000, followed by ECL visualization. Alternatively, affinity-purified Alexa Fluor 680- or IRDye800-conjugated goat anti-IgG antibodies were used as secondary antibodies, followed by scanning and quantitation with Odyssey (LI-COR Biosciences).

**Immunoprecipitation/Phosphatase Assay**—Following immunoprecipitation from denatured cell lysates, Sepharose-bound proteins were washed twice with 1 ml of Nonidet P-40 buffer and four times with 1 ml of phosphatase buffer (25 mM Hepes-NaOH (pH 7.4), 150 mM NaCl, and 0.1 mg/ml bovine serum albumin), divided in half, and pulsed down, and the supernatant was aspirated off. 10-μl reactions composed of 1× phosphatase buffer, 2 mM MnCl\(_2\), and 1 μl of λ-protein phosphatase (New England Biolabs) or 1 μl of \( H_2O \) were then incubated at 30 °C for 45 min with gentle mixing. The beads were washed three times with Nonidet P-40 buffer and resuspended in 25 μl of 2× SDS sample buffer.

**Sucrose Gradient Analysis**—For sucrose gradient analysis, cells were grown to mid-log phase in yeast extract medium at 32 °C. Approximately 2 × 10^8 cells were collected by centrifugation, and native lysates in Nonidet P-40 buffer were prepared as described above. Lysates were layered on 10–30% sucrose gradients prepared in Nonidet P-40 buffer. Gradients were ultracentrifuged at 30,000 rpm for 21 h in a Beckman SW 50.1 rotor. Sedimentation markers (thryoglobin for 19 S and aldolase for 11.3 S) were fractionated on gradients prepared and spun in parallel. Fractions were collected, run on 10% SDS-polyacrylamide gradient gels, and then immunoblotted as described above.

**Purification and Analyses of Lid1-TAP Complexes**—8-liter cultures of lid1-TAP and lid1-TAP hcn1::ura4^+ leu1-32::nmt81hcn1cDNA were grown to log phase, and the tagged proteins were isolated as described (36). The TAP complex was analyzed by direct analysis of large protein complexes/tandem mass spectrometry as described previously (37). However, the TAP complexes prepared from lid1-TAP hcn1::548A and cut9-TAP were analyzed differently in that they were run by three-phase multidimensional protein identification technology (MudPIT) on an LTQ instrument (Thermo Electron Corp.). The three-phase MudPIT was set up as described (38) with the following modifications. Briefly, 12 elution steps were included in the three-phase MudPIT experiments. For each salt elution, 2 μl of ammonium acetate was injected through an autosampler (FAMOS). The concentration of salt used for each step was 0 mM, 10 mM, 25 mM, 50 mM, 100 mM, 200 mM, 300 mM, 400 mM, 600 mM, 800 mM, 1 M, and 5 M, respectively.

**Yeast Two-hybrid Analysis**—The yeast two-hybrid system used in this study was described previously (39). cDNAs of APC subunits were cloned into the bait plasmid pGBT9 and/or the prey plasmid pGAD424 and sequenced to ensure the absence of PCR-induced mutations and that the correct reading frame had been retained. To test for protein interactions, both bait and prey plasmids were cotransformed into S. cerevisiae strain PJ69-4A. β-Galactosidase reporter enzyme activity in the two-hybrid strains was measured using a Galacto-Star™ chemiluminescent reporter assay system (Tropix Inc.) according to the manufacturer’s instructions, with the exception that cells were lysed by glass bead disruption. Each sample was measured in triplicate. Reporter assays were recorded on a Mediator PhL luminometer (Aureon Biosystems).

**Phosphoamino Acid Analysis and Tryptic Peptide Mapping**—\( 32P \)-Labeled Hcn1-Myc\(_6\) or Hcn1-GFP (bound to polyvinylidene difluoride membrane) was used for phosphoamino acid analysis or tryptic peptide mapping as described previously (40, 41).

**In Vitro Kinase Assay**—All bacterially produced recombinant proteins were purified on amunoose beads (for maltose-binding protein (MBP)). Approximately 100 ng of recombinant Cdk1 kinase complex, purified from baculovirus-infected insect cells as described (26), was used to phosphorylate ~1 μg of bacterially produced MBP. Hcn1 in HB15 buffer (25 mM MOPS, pH 7.2, 60 mM β-glycerophosphate, 15 mM p-nitrophenylphosphate, 1% Nonidet P-40, 15 mM EDTA, 15 mM MgCl\(_2\), 1 mM DTT) supplemented with 10 μM unlabeled ATP and 5 μCi of [γ-\( 32P \)]ATP. Reactions were incubated at 30 °C for 30 min and terminated by the addition of sample buffer. Samples were boiled and separated by SDS-PAGE. Coomassie Blue staining and autoradiography were performed for the detection of proteins.

**Site-directed Mutagenesis and Gene Replacement**—Serines within Hcn1 were changed to alanines using a QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. To generate a gene replacement strain, the hcn1-S48A mutation in a genomic clone contained in pIRT2 was transformed into the heterozygous hcn1/S hcn1::ura4^+ diploid strain. Leu^+ diploids were allowed to sporulate, and Leu^+ Ura^- haploid progeny were isolated. These were grown overnight in minimal medium containing uracil and leucine. The following day, 3 × 10^7 cells were plated on minimal medium containing uracil, leucine, and 1 mg/ml 5-fluoroorotic acid. Ura^- Leu^- colonies were then selected. To confirm that the correct gene replacement was present within these cells, the relevant genomic DNA of hcn1 was amplified by whole cell PCR using oligonucleotides hcn1-For2 (5'-ctctctctt-gctttcatccttg-3') and hcn1-Rev (5'-cagcagtagagccaggctg-3'). The PCR product was sequenced directly using oligonucleotide hcn1-For1 (5'-catgttacgaagaaatcctacg-3').

**RESULTS**

To confirm that Hcn1 is a constitutive APC component, the locus encoding hcn1^+ was modified to produce Hcn1-GFP or...
Hcn1-Myc₁₃, and the Myc-tagged allele was combined with cut9-HA₃. In an anti-HA immunoprecipitate from hcn1-Myc₁₃ cut9-HA₃, both Hcn1-Myc₁₃ and Cut9-HA₃ were detected, and conversely, in an anti-Myc immunoprecipitate, both Hcn1-Myc₁₃ and Cut9-HA₃ were detected (Fig. 1A). We next tested whether the association between these proteins is constant during the cell cycle. Small G₂ phase cells of the hcn1-Myc₁₃ cut9-HA₃ strain were collected by centrifugal elutriation and released into fresh medium to obtain a synchronous cell population. Samples were taken every 15 min and used for anti-Myc immunopurification. The amount of Hcn1-Myc₁₃ from every time point was constant, and the amount of co-purified Cut9-HA₃ was constant throughout the entire cell cycle, although its phosphorylation state changed as evidenced by lower mobility forms (Fig. 1B) (20). Also, Hcn1-Myc₁₃ co-sedimented in a sucrose gradient with Cut9-HA₃ (Fig. 1C). These data indicate that Hcn1 is a core component of the APC/C.

To determine whether Hcn1 is essential for viability, hcn¹⁺ was deleted by replacing it with the ura4⁺ gene in a diploid. Tetrad analysis of the correct heterozygous diploids gave rise to two viable Ura− colonies and two nonviable colonies (data not shown), indicating that hcn¹⁺ is essential. To examine the consequence of Hcn1 loss to APC/C function, a conditional null allele of hcn¹⁺ was constructed by placing it under the control of the thiamine-repressible nmt81 promoter (42) in the hcn1::ura4⁺ mutant background. The resultant strain was normal when grown in the absence of thiamine, but depletion of hcn¹⁺ through the addition of thiamine led to cell death. These cells failed to segregate their DNA properly, displaying a “cut” phenotype (Fig. 1D) typical of APC/C mutants (43). These data indicate that Hcn1 is essential for S. pombe viability most likely because of a lack of APC/C activity.

Because Hcn1 loss resulted in a phenotype similar to those of other mutants that affect assembly of the APC/C (20, 26, 44), the integrity of the APC/C complex in the absence of Hcn1 was examined by sucrose gradient sedimentation. When hcn¹⁺ was normally expressed, both Lid1-Myc₁₃ and Cut23-Myc₁₃ sedimented in a peak at ~20 S as expected (Fig. 2A). In contrast, Lid1-Myc₁₃ and Cut23-Myc₁₃ shifted into smaller complexes when expression of hcn¹⁺ was repressed (Fig. 2A). Because Cut23-Myc₁₃ sedimented in a smaller complex than the subcomplex containing Lid1-Myc₁₃, it seemed possible that the loss of Hcn1 split the APC/C into at least two distinct parts. To determine which APC/C subunits remained in the Lid1 subcomplex, we purified Lid1-TAP from the conditional hcn¹⁺ shutoff strain. Compared with the wild-type complex on a silver-stained gel, significant numbers of proteins were diminished in abundance or were absent (Fig. 2B). Mass spectrometric analysis of this complex revealed that Cut4, Apc2, Lid1, Apc5, Apc11, and perhaps Apc15 were present at least 25% of the recovery in hcn¹⁺ cells, but the recovery of other components was reduced more significantly (Table 2). Although this technique is not quantitative, it does suggest that specific components remain associated with Lid1 in the absence of Hcn1, whereas others do not. We concluded that Hcn1 helps link the Lid1-containing subcomplex to other APC/C subunits.

To determine which component(s) interacted directly with Hcn1 to bridge the Lid1-TAP subcomplex to other subunits, we performed a series of directed two-hybrid analyses between Hcn1 and other APC/C components with only Cut4 and Apc15 not included in the analyses. Hcn1 interacted with only Cut9.
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FIGURE 2. The APC is disassembled by deletion of Hcn1. A, native lysates from the lid1-Myc13 (KGY1336), cut23-Myc13 (KGY33), lid1-Myc13, cut23-Myc13, hcn1-548A-GFP (KGY6158) strains and conditional hcn1 shutoff strains that had been growing in the presence of thiamine for 18 h and harboring lid1-Myc13 (KGY3590) or cut23-Myc13 (KGY3658) or cut23-Myc13 (KGY4581) were resolved on a sucrose gradient. Fractions were collected from the bottom (first lane) and immunoblotted with antibody 9E10 to detect Lid1-Myc13 or Cut23-Myc13. The peaks of thyroglobulin (19 S) and aldolase (11.3 S) collected from parallel gradients are indicated. B, the Lid1-TAP complex was purified from the wild-type strain (KGY3590) or the hcn1 shutoff strain (KGY4578) grown to log phase in the presence of thiamine. Proteins co-purifying with Lid1-TAP were revealed by silver stain. C, strain PJ206 was cotransformed with the bait plasmid pGBT9 and the prey plasmid pGAD424 in which APC/C cDNAs were cloned as indicated or not and then screened for their ability to grow on His- and Ade-deficient (double selection) plates. Streaks are shown in the right panels, with vector control transformants on the left side of plate and test vector transformants on the right. Based on the results from β-galactosidase assay, each interaction is shown as a bar graph. Relative light units of β-galactosidase activity are designated in parentheses.

TABLE 2
Effect of Hcn1 on Ld1-TAP composition

| Component | Mass (kDa) | lid1-TAP mts3-1 | lid1-TAP mts3-1 hcn1-ara4 \( \text{leu}3-32::\text{mnt81} \) hcn1 | lid1-TAP mts3-1 hcn1-S48A* |
|-----------|------------|----------------|---------------------------------|----------------------------|
| Cut4      | 165.4      | 41             | 23                             | 80                         |
| Apcl      | 80.5       | 20             | 14                             | 43                         |
| Nucl      | 76.2       | 32             | 6                              | 51                         |
| Ld1       | 82.6       | 19             | 10                             | 44                         |
| Apcl5     | 85.3       | 32             | 19                             | 43                         |
| Cut9      | 75.9       | 22             | 1                              | 53                         |
| Cut23     | 60.4       | 18             | 4                              | 33                         |
| Apcl10    | 21.5       | 4              | 0                              | 10                         |
| Apcl11    | 10.5       | 5              | 2                              | 5                          |
| Hcn1      | 9.1        | 4              | 1                              | 8                          |
| Apcl13    | 15.6       | 4              | 0                              | 10                         |
| Apcl14    | 12.1       | 5              | 0                              | 10                         |
| Apcl15    | 15.7       | 2              | 1                              | 6                          |

* These data were acquired on the more sensitive LTQ mass spectrometer.

(Fig. 2C), offering an explanation for the isolation of hcn1+ as a high copy suppressor of the cut9-665 mutation (20). In addition to testing Hcn1 two-hybrid interactions, we examined pairwise interactions among other APC/C subunits, again with the exception of Cut4 and Apcl5. The following interactions were the only ones detected: Apcl2 showed a strong interaction with Apcl11; Nucl2 interacted with Apcl10; Ld1 interacted with Apcl5; and Cut23 interacted with Apcl13 (Fig. 2C). These interactions are consistent with the mass spectrometric data of the Ld1-TAP complex obtained above, as well as with other data regarding subcomplexes of the APC/C observed in other organisms (45, 46).

Many APC/C components are phosphoproteins, and phosphorylation of the APC/C during mitosis positively regulates its activity (9, 10, 23, 25). To examine Hcn1 phosphorylation during mitosis, we used two different methods of arresting cells in a mitotic state, one using Mph1 overexpression and another using the mts3-1 mutant. Mph1 is a spindle checkpoint kinase; its overexpression mimics activation of the checkpoint and imposes a metaphase arrest (47). mts3+ encodes one subunit of the 26 S proteasome, and its mutant allele (mts3-1) is defective in the metaphase/anaphase transition at non-permissive temperature (48). Hcn1-Myc13 from metaphase-arrested cells, but not from asynchronous cells, showed a reduced mobility on gels, which was eliminated by phosphatase treatment (Fig. 3A), indicating that the shift was due to phosphorylation. The shift corresponding to altered phosphorylation was also detected in a synchronous population of hcn1-GFP cells as they progressed through mitosis by the appearance of a single more slowly migrating band (Fig. 3B; see Fig. 4A). The hyperphosphorylated state of Hcn1-GFP peaked prior to septation (Fig. 3B). Phosphorylation of Hcn1-Myc13 and Hcn1-GFP was also detected by in vivo labeling with [32P]orthophosphate in cells arrested with the mts3-1 mutation (Fig. 3C). Phosphoamino acid analysis of in vivo labeled Hcn1-Myc13 (Fig. 3D) and Hcn1-GFP (data not shown) revealed that Hcn1 was phosphorylated exclusively at serine residues. Hcn1 has eight serines, and one (Ser48) corresponds to the strict consensus site for Cdk phosphorylation (SPXK). Thus, we tested whether recombinant Cdc2-Cdc13 kinase (the S. pombe Cdk1 complex) could phosphorylate
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MBP-Hcn1 or two mutants of MBP-Hcn1 in which Ser<sup>48</sup> or Ser<sup>69</sup> was altered to alanine by site-directed mutagenesis. Although MBP-Hcn1 and MBP-Hcn1-S69A were phosphorylated by Cdc2-Cdc13, MBP-Hcn1-S48A was not (Fig. 3E), suggesting that Ser<sup>48</sup> is the sole site phosphorylated by Cdc2-Cdc13 in vitro.

Phosphopeptide maps of MBP-Hcn1 labeled in vitro by Cdc2-Cdc13 gave rise to two spots that are likely related and that arose from variable tryptic digestion within the SPEKKK sequence (Fig. 3F, right panel). Two similar major spots were also produced from in vivo labeled Hcn1-GFP (Fig. 3F, left panel) and Hcn1-Myc<sub>13</sub> (data not shown). These two major spots comigrated when equal counts of the two samples were mixed (Fig. 3F, MIX), suggesting that Hcn1 is phosphorylated in vivo at Ser<sup>48</sup> during mitosis.

Interestingly, there was a minor constellation of spots visualized in Hcn1-Myc<sub>13</sub> samples that was absent in the Hcn1-GFP sample, and two pieces of evidence suggest that these arose from phosphorylation within the Myc<sub>13</sub> epitope itself. First, they comigrated with phosphopeptides generated from the otherwise unrelated Sop2-Myc<sub>13</sub> protein; and second, they persisted in labeled Hcn1-Myc<sub>13</sub> lacking all of its eight serine residues (data not shown).

To verify that Ser<sup>48</sup> is the single site of Hcn1 phosphorylation, we generated a strain in which Ser<sup>48</sup> was replaced with alanine (hcn1-S48A) at the hcn1<sup>+</sup> genomic locus (see “Experimental Procedures”). The locus was then tagged with GFP or Myc<sub>13</sub> to allow detection of the mutant protein. Both tagged and untagged hcn1-S48A strains were viable, indicating that phosphorylation of Hcn1 at Ser<sup>48</sup> is not essential. Like Hcn1-Myc<sub>13</sub>, Hcn1-GFP migrated as multiple bands during mitosis, and the upper band could be eliminated by phosphatase treatment (Fig. 4, A and B). Mutation of Ser<sup>48</sup> abolished the upper mobility form of Hcn1-GFP (Fig. 4, A and B), indicating that the gel shift was due to this phosphorylation event. Labeling of Hcn1-S48A-GFP in vivo with [<sup>32</sup>P]orthophosphate revealed that no phosphate was incorporated into the protein relative to Hcn1-GFP (Fig. 4C). Taken together, our data suggest that Ser<sup>48</sup> is the only significant in vivo phosphorylation site within Hcn1.

The hcn1-S48A mutant strain described above was used to determine whether Hcn1 phosphorylation affects APC/C activity and/or cell cycle progression. Defects in S. pombe APC/C function have manifested themselves previously with the development of a cut phenotype in which 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 (43). This phenotype was not observed in hcn1-S48A cells (Fig. 5A). Instead, many cells in the culture (>30%) showed cell separation defects resulting in doublets or chains of cells, particularly at 36 °C (Fig. 5A). These doublets, arising from incomplete cell division, were separated partially digested with trypsin while bound to polyvinylidene difluoride membrane.

Equal cpm of tryptic peptides were spotted separately or mixed together (MIX) and resolved in two dimensions on cellulose thin-layer plates by electrophoresis at pH 1.9 with the anode on the left and by ascending chromatography.
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![Diagram](image)

**FIGURE 4. Hcn1 is phosphorylated at Ser**

A lysate from the indicated strains was subjected to immunoprecipitation with anti-GFP antibody and treated with λ-protein phosphatase or buffer alone. Immunoprecipitates were resolved by SDS-PAGE, blotted, and probed with antibody to the GFP epitope.

**B**

Wild-type and hcn1-S48A-GFP strains were transformed with pREP1 or pREP1mph1, and transformants were grown in the absence of thiamine for 18 h. Protein lysates were prepared and subjected to immunoprecipitation with anti-GFP antibody. The immunoprecipitates were resolved by SDS-PAGE, and GAPD-tagged proteins were detected by immunoblotting. C, the indicated proteins in a mts3-1 background (strains KGY1834 and KGY5632) were labeled with [32P]orthophosphate during a 4-h shift to the non-permissive temperature, and protein lysates were immunoprecipitated with anti-GFP antibody. The immunoprecipitates were resolved by SDS-PAGE, and labeled proteins were detected by autoradiography.

by sonication and completely by treatment with cell wall-lysing enzymes (data not shown), indicating that independent daughter cells remained connected by undissolved cell wall material. The mutation was recessive because the phenotype of an hcn1+/hcn1-S48A diploid was wild type (data not shown). We also generated a strain in which Ser48 was replaced with aspartic acid (hcn1-S48D) in an effort to mimic constitutive phosphorylation. This strain was morphologically wild type (data not shown) and had a normal septation index (13%) at all temperatures. We concluded that the cell separation defect is probably due to a lack of Hcn1 phosphorylation.

To determine whether the delayed cell separation in the hcn1-S48A strain was due to reduced APC/C function, we examined genetic interactions between hcn1-S48A and other APC/C mutations. Although no synthetic interactions were detected with cut4-s33, cut9-665, apc14Δ, or apc15Δ (data not shown), hcn1-S48A reduced the restrictive temperatures of both lid1-6 and nuc2-663 (Fig. 5B), suggesting that the lack of Hcn1 phosphorylation compromised APC/C function.

As mentioned above, because of a block in Cut2 (securin) degradation, other S. pombe APC/C mutants display a cut phenotype. Although this phenotype was not observed in the hcn1-S48A strain, the genetic interactions described above prompted us to examine hcn1-S48A cells more carefully for defects typi-
pombe APC/C, Hcn1. Our results indicate that Hcn1 is essential for cell viability, APC/C integrity, and proper APC/C regulation. Moreover, our analysis of Hcn1 phosphorylation indicated a specific role for the phosphorylation of this subunit late in the cell cycle.

We found that, in the absence of hcn1, the APC/C is split into at least two smaller subcomplexes. The first contains Cut4 (APC1), Lid1 (APC4), Apc2, Apc5, and Apc11. The second consists of the tetratricopeptide repeat proteins and other components (this study and data not shown). Similar subcomplexes have been purified previously from human and S. cerevisiae cells (45, 46, 53). One subcomplex consisting of APC1, APC2, APC4, APC5, and APC11 was found to interact with a ubiquitin conjugating enzyme and to assemble polyubiquitin chains, but could not conjugate these chains to a substrate (45). A stable subcomplex of the tetratricopeptide repeat proteins Cdc16, Cdc27, and Apc9, as well as of Swm1/Apc13 and Cdc26, has been detected in S. cerevisiae (46, 53). Furthermore, in cdc26Δ cells, the amounts of Cdc16, Cdc27, and Apc9 were reduced in immuno-precipitates of Apc2 (53), implying that, like Hcn1, Cdc26 is required for incorporation of a set of subunits into the APC/C especially at increased temperature. These subunits are implicated in binding activator and substrate proteins (54, 55). Putting our purification and mass spectrometric data together with our two-hybrid analyses, we have arrived at a model of APC/C organization that is very consistent with the detailed architecture proposed recently for the S. cerevisiae APC/C by Thornton et al. (46).

However, we found one additional interaction that deviates from this proposal: an interaction between Apc10 and Nuc2. Human APC10 was also shown to bind APC3/CDC27 (56). It is possible that Apc10 has more than a single interaction surface on the APC/C and is present in multiple copies. In support of this possibility, it has been reported that S. cerevisiae Apc10 is present in multiple copies per APC/C complex (57).

While examining the associations of Hcn1, we recognized that Hcn1 is post-translationally modified and confirmed that it is phosphorylated during mitosis at a single site, Ser⁴⁸. Ser⁴⁸ conforms to a strict consensus Cdk1 site, and Hcn1 Ser⁴⁸ is phosphorylated in vitro by Cdk1. Thus, Hcn1 is most likely phosphorylated by Cdk1, although we cannot exclude the possibility that other kinases target this site. Given the interaction of Hcn1 with Cut9 and the overall organiza-
Hcn1 Phosphorylation Affects APC/C Function

A

\[
\begin{align*}
\text{hcn1} & \quad \text{hcn1-S48A} \\
\text{Ace2-\textbf{myc}}_{13} & \quad \text{Hcn1-GFP} \\
\text{Cdk1} & 
\end{align*}
\]

B

\[
\begin{align*}
\% & \text{ doublets} \\
p\text{REP3X} & \quad p\text{REP3X}\text{ace2} \\
\end{align*}
\]

FIGURE 6. Ace2 is limiting in hcn1-S48A cells. A, protein lysates from ace2-Myc13, hcn1-GFP (KGY1565) and ace2-Myc13, hcn1-S48A-GFP (KGY1566) cells grown at 36 °C were probed for Ace2, Hcn1-GFP, and Cdk1 levels. Signals were quantified with Odyssey. B, hcn1-S48A-GFP cells (KGY477S) were transformed with pREP3X or pREP3Xace2. Transformants were grown at 36 °C to mid-log phase and stained with aniline blue to score the percentage of doublets in the population. Five sets of 100 cells were counted in each case, and S.D. values are given by error bars.

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