INHIBITION OF THE CDH1-DEPENDENT ANAPHASE-PROMOTING COMPLEX BY ACM1

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ABSTRACT

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Inhibition of the Cdh1-Dependent Anaphase Promoting Complex by Acm1
(Under the direction of Dr. Christoph H. Borchers)

Untimely progression through the cell cycle can lead to catastrophic and irreversible genetic anomalies. Therefore, the cell cycle must be tightly controlled and proceed unidirectionally. Cell cycle control is due in part to the targeted ubiquitin-mediated proteolysis of regulatory proteins. The anaphase-promoting complex (APC) is an essential E3 ubiquitin ligase responsible for catalyzing the proteolysis of regulatory proteins in the cell cycle. Cdh1 is a co-activator of the APC aiding in the onset and maintenance of G_1 phase, while phosphorylation of Cdh1 at the end of G_1 phase by cyclin dependent kinases assists in the inactivation of APC^{Cdh1}. Here, we suggest additional components are involved in the inactivation of APC^{Cdh1} independent of Cdh1 phosphorylation. These components were identified as Acm1, Bmh1, and Bmh2 in complex with Cdh1. Acm1 is cell cycle regulated and its levels are highest in S phase when APC^{Cdh1} is inactive. Complex formation is dependent on the presence of phosphorylated Acm1. Acm1 alone is capable of inhibiting ubiquitination of Clb2 and Pds1 *in vitro* while overexpression of Acm1 rescues the lethality caused by overexpression of the constitutively active Cdh1 alanine phospho-mutant. Acm1 inhibition is specific to APC^{Cdh1} as it does not inhibit APC^{Cdc20} *in vitro*. While partially

phosphorylated Cdh1 can still activate the APC, the addition of Acm1 decreases Clb2 ubiquitination when using either phosphorylated or nonphosphorylated Cdh1.

Cdh1 contains three consensus D-box regions (R-X-X-L) and one KEN box region. Mutations of the 3rd D box and the KEN box of Cdh1 abolish its interaction with Acm1. The same mutant is unable to inhibit APC^{Cdh1} activity *in vitro* and *in vivo*. Although full length Acm1 does not appear to be ubiquitinated while serving as an APC inhibitor, truncations of both the N-terminus and C-terminus of Acm1 convert the protein to an APC^{Cdh1} substrate. We conclude Acm1 acts as a competitive inhibitor and blocks substrate binding to the Cdh1, thereby inactivating APC^{Cdh1}. Together, these findings suggest a novel means of APC^{Cdh1} regulation exists that is independent of Cdh1 phosphorylation in yeast through inhibition by Acm1.

DEDICATION

In memory of Anne Dial who has always been and will forever be my inspiration

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CHAPTER 1

Introduction

Overview

Defects in the regulation of the eukaryotic cell cycle are responsible for cell death, uncontrolled proliferation, and genetic anomalies (1-4). Therefore, progression of the cell cycle must be tightly controlled and proceed unidirectionally to prevent genetic catastrophe. Cell cycle control is largely due to targeted proteolysis of regulatory proteins, specifically by ubiquitin-mediated proteolysis. The anaphase-promoting complex (APC) is an essential E3 ubiquitin ligase responsible for catalyzing proteolysis of key regulatory proteins in the cell cycle. The APC is closely regulated by phosphorylation and protein-protein interactions. The APC has two co-activators in mitosis, Cdc20 and Cdh1, whose presence is necessary for APC activation and control substrate specificity. Cdc20 and Cdh1 are also tightly regulated, modulating APC activity on additional levels. The focus of this research examines the eukaryotic cell cycle and its regulation, specifically control of APC^{Cdh1} activity by the negative regulator Acm1 in mitotic cells.

The Eukaryotic Cell Cycle

The main function of the cell cycle is to replicate the cell's genome and allocate this information to two daughter cells. These highly ordered events are described in four major phases, gap 1 (G_1), synthesis (S), gap 2 (G_2), and mitosis (M). Gap 1 allows for cell growth in order for cells to double their mass of organelles and proteins that are required for DNA synthesis. External and internal environmental factors are also closely

monitored during G_1 phase for cells to certify all conditions are right for the major task of DNA synthesis in S phase (5). The length of G_1 can vary depending on extracellular conditions and signals. If conditions are unfavorable, such as lack of proper nutrients for cell growth, cells will enter a specialized resting state known as G_0. Once conditions are favorable for cell growth, cells progress through G_1 or G_0 past a commitment point towards the end of G_1 known as Start in yeast (5). When cells reach Start they are then devoted to proceeding with DNA replication. DNA replication or S phase involves the controlled synthesis of the chromosomal DNA. Cells then enter G_2 phase. Much like G_1, cells in G_2 continue to monitor cellular conditions prior to entering mitosis. G_1, S phase, and G_2 together make up interphase. Mitosis, or M phase, follows G_2 and is the process of dividing the replicated DNA from S phase into two daughter cells. M phase is made up of six stages, including prophase, prometaphase, metaphase, anaphase, telophase, and cytokinesis. Within M phase, the replicated DNA is packaged into sister chromatids which align at the center of the cell and microtubules connect the sister chromatids to spindle poles (5). At the metaphase to anaphase transition, the microtubules shorten and the spindle poles move apart pulling the sister chromatids away from one another for incorporation into each daughter cell. Untimely progression through this stage can be problematic. For example, early onset of mitosis before complete DNA replication takes place or before microtubules are properly attached to sister chromatids can lead to aneuploidy (6). Other crucial events include the exit from mitosis and the initiation of DNA replication at the G_1/S phase transition. Control of these important events in the life of a cell is largely controlled by cyclin-dependent kinases (CDKs), which consist of a catalytic protein kinase subunit and a regulatory cyclin subunit.

In *Saccharomyces cerevisiae*, a single CDK known as Cdc28 is able to regulate multiple cell cycle transitions in large by associating with different cyclins at specific times during the cell cycle. In G_1, Cdc28 requires three G_1 cyclins, Cln1-3 while S phase is mediated by Clb5-6 and Clb1-4 govern mitosis (7).

Phosphorylation of a variety of proteins by CDKs is required for cell cycle progression. One example includes phosphorylation of the negative cell cycle regulator Sic1 (8). Sic1 is an inhibitor of B-type cyclin (Clb)-CDK complexes preventing S phase entry and is stable until the G_1/S phase transition when it is phosphorylated by G_1 cyclin (Cln)-CDK activity triggering its proteolysis (9,10).

CDK activity can be regulated in three different ways including phosphorylation of the kinase subunit, controlling protein levels through mRNA or protein degradation, and by activation or inactivation of CDK inhibitors (CKI) (7). After each cell cycle phase is completed, down-regulation of the CDK activity responsible for the previous phase maintains tight control over the cell cycle transitions. Just as early onset of mitosis before DNA replication is completed is disastrous for the cell, so are additional rounds of DNA replication before proper chromosome segregation. Because of the great importance of proper cell cycle progression, eukaryotic cells have developed extensive mechanisms for CDK inactivation. One important way cells govern CDK down-regulation is through the destruction of cyclin subunits. During both the G_1/S phase and metaphase to anaphase transition, CDK activity is shutoff through the ubiquitin-mediated proteolysis of cyclins (11).

Ubiquitin-mediated Proteolysis.

The driving force for forward progression and the primary mechanism by which each cell cycle stage changes is through ubiquitin-mediated proteolysis of regulatory proteins. Ubiquitin-mediated proteolysis is the most common way by which cells degrade proteins. Ubiquitin is a small 8 kDa protein present in all eukaryotes. Three distinct enzymatic activities occur to polyubiquitinate proteins resulting in their recognition and destruction by the proteasome. First, the ubiquitin activating enzyme (E1) covalently binds the carboxyl terminus of the highly conserved ubiquitin protein via a thioester linkage. The ubiquitin is then transferred to one of several ubiquitin-conjugating enzymes (E2). The ubiquitin-protein ligase (E3) directs the transfer of the ubiquitin from the E2 to a lysine residue on a target protein substrate. This process is repeated several more times so additional ubiquitin proteins are added to the first, creating a polyubiquitin chain that serves for recognition by the 26s proteasome (12). The E3 enzyme is of great importance because it is responsible for directing ubiquitin conjugation to specific substrates at specific times.

The list of cell cycle regulated substrates that are targeted for ubiquitin-mediated proteolysis is extensive. In addition to cyclins, an important example is the precisely timed degradation of the mitotic inhibitor securin. Securin is bound to a protease known as separase. Upon degradation of securin, separase is freed to cleave cohesin, a protein which holds sister chromatids together in metaphase (13). Once cohesin is cleaved, sister chromatids are allowed to separate and anaphase onset occurs. Other notable examples of cell cycle regulated proteins include Sic1 in *Saccharomyces cerevisiae* and $p27^{Kip1}$ in humans, both CDK inhibitors at the G_1/S transition. Most regulated proteolysis can be attributed to two ubiquitin ligases in the cell cycle. One is the Skp1/Cul1/F-Box (SCF)

complex, required for the G_1 to S phase transition and initiation of DNA replication (14). The other is the anaphase-promoting complex (APC), required for the metaphase to anaphase transition, exit from mitosis, and the onset and maintenance of G_1 (15).

The Skp1-Cul1-F-box protein complex and the Anaphase-Promoting Complex

The SCF complex and the APC are members of the cullin based subfamily of E3 ligases within the larger RING-finger-type E3 family. The RING-finger-type E3s are one of four types of E3 ligases including HECT-type, U-box-type, and PHD-finger type all based on their specific structural motif. Of these families, the RING-finger E3 is the largest. The SCF complex consists of three main components, RBX1 (Ring-finger protein), CUL1 (scaffold protein), SKP1 (adaptor protein), and three interchangeable receptor proteins known as SKP2, FBW7, and β-TRCP (16). The receptor proteins play a role in recognition of the E3 substrate and are thought to be involved in cell cycle control.

The APC is a RING finger E3 ubiquitin ligase similar to the SCF. It is a very large complex (1700 kDa) consisting of 13 subunits in yeast and 11 in humans (17-20). Its main function is suggested to bring the E2 and the substrate close enough together to allow for a direct transfer of ubiquitin. The subunit APC2 (scaffold protein) consists of a cullin-homology region and is suggested to provide a platform for proper alignment of the E2 and substrate (21). The subunit APC11 contains a Zn^{2+}-binding motif referred to as the RING-H2 finger (22). APC2 and APC11 are homologous to the functional core of the SCF ubiquitin ligases, and recent studies have shown they are essential for

ubiquitination activity (23,24). Ubiquitin ligase activity of the APC has been shown to require the presence of one of two co-activator proteins in mitosis known as Cdc20 or Cdh1 (25-33). The co-activators interact with the APC at different times during the cell cycle with Cdc20 activating the APC from early to late M phase and Cdh1 from late M phase through G_1 (27,29,34). The co-activators Cdc20 and Cdh1 are characterized by a C-terminal WD-40 domain as well as a C-box and IR-tail (23,35,36). The C-box and IR-tail mediate the co-activator binding to the APC while the WD-40 domains recognize APC substrates through specific substrate regions called KEN boxes and D boxes (33,37,38). Mutation of the WD-40 region of Cdh1 in complex with the APC disables binding to substrates (38). The IR-tail of Cdh1 interacts with the TPR-domain protein Cdc27 on the APC complex (23,38). Apc2 appears to interact with the C-box domain of Cdh1 either through Apc2 directly or indirectly by an Apc2 binding partner or Apc11 (24). Catalysis of the ubiquitination reactions may be enhanced by the proximity of Cdh1 to the catalytic subunits Apc2 or Apc11 and the E2 enzyme (24). These findings support the role of the co-activators as necessary components for substrate recognition and APC activation.

APC subunits have also been implicated in substrate recognition. Reports have shown APC substrates bind directly to the APC but binding is dependent on the presence of Cdh1 or Cdc20 (36,37,39). When the APC subunit Doc1 is deleted, APC can no longer bind to substrates and ubiquitinate them despite the APC binding to Cdh1 or Cdc20 (36,40). Crystal structures of Doc1 have shown a globular domain with a surface predicted to interact with a ligand although the ligand is unknown (41). Mutation of this region disables APC ubiquitination activity (42). Therefore, evidence suggests Doc1

may play a role in substrate binding.

The function of the numerous other APC subunits is less clear. Swm1, Mnd2, Apc4, Apc9, and Cdc26 in *S. cervisae* do not share any significant homology with proteins of known function so their purpose is not known at this time. Deletion of Cdc26 has been shown to result in a decreased association of Apc9, Cdc16, and Cdc27 suggesting Cdc26 stabilizes other APC subunits (43). Other subunits may also play a role in stabilization of the APC complex. Cdc27 association is diminished upon deleting APC9 (43). Furthermore, deletion of SWM1 causes disassociation of Cdc16, Cdc26, Cdc27, and Apc9 from the APC (44). However, questions concerning the mechanism of catalysis by the APC remain, including the function of the other numerous subunits and how and where substrates interact with the APC.

The APC has essential functions within the cell cycle including mitotic progression, mitotic exit, and preparation for DNA synthesis that are dependent on the ability of APC to selectively recognize substrates at precise moments. One important role APC plays in the cell cycle is in the initiation of cyclin B (Clb1,2 in budding yeast) proteolysis. Destruction of B type cyclins begins in metaphase and is necessary for CDK inactivation and mitotic exit. Once cyclin B is degraded, Cdk1 is rendered inactive allowing phosphatases to dephosphorylate Cdk1 substrates. This leads to disassembly of the mitotic spindle, decondensation of chromosomes, reformation of the nuclear envelope, and formation of the cytokinetic furrow (11). Low CDK activity during telophase and G_1 is required for formation of pre-replicative complexes on replication origins where DNA polymerases initiate DNA synthesis. B type cyclin degradation is mediated by APC^{Cdc20} while cyclin B levels are kept at bay by APC^{Cdh1} during G_1.

Interestingly, lethality has been reported in cells with *sic1Δ* and *cdh1Δ* presumably because of the cell's inability to inactivate cyclin B-CDK activity at mitotic exit (25,45). The B type cyclins Clb5 and Clb6 are also required for CDK activity and S phase entry but are not APC substrates. Therefore, the APC maintains low CDK activity when pre-replicative complexes are forming but allows CDK activation by Clb5 and Clb6 for DNA synthesis thereby allowing origins of replication to only fire once per cell cycle.

As the name "anaphase-promoting complex" implies, another important role of the APC is in the advancement of metaphase to anaphase. Securin, a co-chaperone and inhibitor of separase, is ubiquitinated by APC^{Cdc20} triggering its proteolysis. Separase is then allowed to cleave the Scc1 subunit of cohesin, a complex that holds sister chromatids together. The cleavage of cohesin allows the disassociation of the sister chromatids thereby promoting anaphase.

A large number of APC substrates have been reported (46). The orchestrated activation of the APC for the timely degradation of substrates is of the utmost importance for the cell. Therefore, highly evolved means of regulation are in place to control APC activity during the cell cycle window to mediate the ubiquitin-mediated destruction of these substrates.

Regulating APC Activity

The APC appears to be a very stable complex that is constitutively present throughout the cell cycle although its activity fluctuates (47,48). One way of controlling APC activity is through phosphorylation of APC subunits. For example, APC from

human mitotic extracts contain hyperphosphorylated APC1, CDC16, CDC23, and CDC27 (47). During mitosis in budding yeast, the Cdc16, Cdc23, and Cdc27 subunits are also hyperphosphorylated (49). Mutations in the CDK sites of these subunits resulted in reduced mitotic activity. Kinases responsible for phosphorylation of the APC subunits have also been identified. For instance, during mitosis CyclinB/Cdc2 (Clb2/Cdc28 in yeast) kinase activity is required for APC activation (49). It is suggested that the family of Polo-like kinases phosphorylate APC subunits and aid in its activation (50). In contrast to CyclinB/Cdc2 and Polo kinases, protein kinase A restricts APC activity by phosphorylating core subunits (50,51). Other kinases involved in activation or inactivation of the APC are likely to exist.

The APC catalyzes ubiquitin-mediated proteolysis of substrates at highly specific times within the cell cycle that allows for mitotic progression, exit from mitosis, and the onset and maintenance of G_1. APC substrate specificity must change throughout the cell cycle in order for these events to take place. For example, the anaphase inhibitor Pds1 (securin) is targeted for proteolysis by APC in metaphase, whereas several mitotic cyclins are degraded after anaphase (13,52). Other substrates such as the S-phase promoting factor Dbf4 are targeted for destruction in G_1 (53). Substrate specificity is regulated in large by the recruitment of co-activator proteins that associate with particular substrates throughout the cell cycle. The co-activators Cdc20 and Cdh1 function to bind specific substrates and are subsequently recruited to the APC where the substrate is poly-ubiquitinated for degradation by the proteasome.

The co-activators interact with specific substrates at specific times so they themselves must be regulated. Phosphorylation of Cdc20 and Cdh1 is an important

control mechanism for both APC^{Cdc20} and APC^{Cdh1} complexes. In some instances, phosphorylation of Cdc20 appears to be necessary for activity of APC^{Cdc20} but not in others (28,54). Although Cdc20 is transcribed and translated during S phase and G_2, it can only activate the APC in mitosis when certain APC subunits are phosphorylated (49,55,56). Phosphorylation of Cdh1 by the cyclin-dependent kinase (CDK), Clb5-Cdc28, inhibits its interaction with the APC (28,54,57). Cdh1 remains hyperphosphorylated throughout S, G_2, and M phase until a drop in CDK activity is triggered by Cdc14 phosphatase (34,58). Therefore, APC^{Cdc20} is active during early mitosis, whereas APC^{Cdh1} can only become activated after APC^{Cdc20} decreases CDK activity through cyclin destruction allowing dephosphorylation of Cdh1 by Cdc14 phosphatase and subsequent activation of APC^{Cdh1}. APC^{Cdc20} is then inactivated through APC^{Cdh1} mediated proteolysis of Cdc20 (34,59,60). APC^{Cdh1} maintains G_1 phase until it is inactivated at the G_1/S transition by S phase CDKs (34). The main S phase cyclin that activates CDKs in budding yeast is Clb5. Clb5 is an APC^{Cdc20} substrate whose levels are low during mitosis (61). Once APC^{Cdc20} is inactivated, Clb5 accumulates and activates Cdc28 allowing the CDK to phosphorylate Cdh1, preventing its interaction with the APC.

While phospho-regulation of co-activators plays an important role in controlling APC activity, interactions between the co-activators and other proteins are also important for their regulation. In vertebrates, a second APC^{Cdh1} inhibition mechanism is in place. The early mitotic inhibitor-1 (Emi1) is expressed at the G_1/S phase transition and competitively inhibits the binding of substrates to APC^{Cdh1} by acting as a pseudosubstrate (62,63). The spindle checkpoint proteins Mad2p and Mad3p/BubR1p bind and inhibit Cdc20 in a substrate specific manner to arrest the metaphase to anaphase transition until

all chromosomes have properly attached to the mitotic spindle (64-68). Emi1 has also been shown to inhibit APC^{Cdc20} as a proposed early mitotic inhibitor before the activation of the spindle assembly checkpoint (69,70). The tumor suppressor protein RASSF1A regulates the timing of mitotic progression by inhibiting the APC^{Cdc20} complex (71). Still others, such as the S-phase checkpoint proteins Mec1p and Rad53 are shown to repress Cdc20 accumulation in S-phase to prevent premature mitotic onset (72). Relocalization of Cdh1 from the nucleus to the cytoplasm by the exportin Msn5 has been shown to occur at the end of G_1 before S phase and remains in the cytoplasm between S phase and the end of mitosis presumably as a redundant mechanism ensuring efficient inactivation (73). Clearly, multiple inhibitory mechanisms are in place to control APC activity because it is crucial to the cell cycle proceeding unidirectionally.

The goal of this research project was to identify new regulatory mechanisms of the anaphase promoting complex through the APC co-activator Cdh1. With the advent of highly sensitive tools for analyzing proteins and peptides such as mass spectrometry, we have the capability to identify potentially novel interacting proteins of the anaphase promoting complex. Before the inception of this work illustrated in the following chapters, no protein inhibitors of APC^{Cdh1} activity had been identified in yeast.

The APC and cancer

Deregulation of cell cycle control is fundamental to cancers. Hence, studying APC regulation is of great clinical importance. Although the APC is not linked to cancer as notably as the SCF, it can be expected that deregulation of either Cdc20 or Cdh1 could

have devastating effects on cell proliferation. Cdc20 overexpression has been linked to pancreatic cancer while its upregulation has been shown in lung and gastric cancer patients (74,75). The spindle assembly checkpoint proteins Mapd2p, Mad3p, and BubR1 which inhibit APC^{Cdc20} activity, prevent precocious segregation of chromosomes leading to aneuploidy, a common form of genetic instability found in cancers (76). Securin, an APC^{Cdc20} substrate is commonly overexpressed in human tumors (77,78). Cdh1 expression is reduced during the malignant progression of a murine B-lymphoma cell line, while the overexpression of Cdh1 suppressed tumor formation (79). Mutations in APC subunits have also been linked to cancer. Mutations in Apc6 and Apc8 have been described in human colon cancer cells (80). The mutation of Apc8 leads to the misregulation of cyclin B levels whose overexpression leads to genomic instability found in colon cancer (80). Other APC substrates have been tied to cancer including the kinases Plk1 and Aurora A. Plk1 controls mitotic entry and cytokinesis and is overexpressed in several tumor types (81). Levels of Aurora A, whose role involves aiding centrosome duplication and separation as well as spindle assembly, are increased in bladder, pancreatic, and ovarian cancers (82-84). Plk1 and Aurora A levels are both mediated by APC^{Cdh1} further suggesting misregulation of APC can lead to tumorigenesis.

Our knowledge of the mechanisms of APC regulation is steadily increasing, leading to the discovery of what roles these mechanisms may play in the development of cancer. The ultimate goal of studying E3 ligases like the APC in cell cycle control is to develop cancer therapeutic treatments that specifically target these enzymes. Some progress has been made in this arena including a small molecule known as 2-methoxyestradiol (2ME2). In collaboration with Nancy DeMore, we have shown 2ME2

to inhibit APC activity through the regulation of genes involved in the spindle assembly checkpoint, resulting in a G_2/M phase arrest (85). Currently, 2ME2 is in phase I/II clinical trials for breast cancer treatment.

Summary

Proper control of the eukaryotic cell cycle is crucial to preventing genetic catastrophes in the cell. The anaphase-promoting complex is an important cell cycle regulating E3 ubiquitin ligase aiding in mitotic progression and a stable G_1 phase. Defects in APC regulation have detrimental consequences leading to tumorigenesis. Our research focuses on the regulation of the APC co-activator Cdh1 during the cell cycle. Specifically, this work concentrates on the discovery and characterization of a novel APC^{Cdh1} inhibitor in budding yeast known as Acm1. We discovered an uncharacterized protein known as Acm1 in a proteomic screen of Cdh1 associated proteins. Along with Acm1, the 14-3-3 proteins Bmh1 and Bmh2 were identified. Acm1, Bmh1, and Bmh2 form a complex with Cdh1 although Acm1 alone is sufficient for APC^{Cdh1} inhibition. The results of this work have determined Acm1 is an APC^{Cdh1} specific inhibitor, acting independently of Cdh1 phosphorylation as an overlapping inhibitory mechanism. Acm1 mimicks an APC substrate and inhibits APC^{Cdh1} activity by blocking substrate binding to Cdh1. The results of this work provide further insight into the complex nature of APC regulation. The discovery and characterization of this novel inhibitory mechanism is outlined in the following chapters.

CHAPTER 2

Inhibition of APC^{Cdh1} activity by Cdh1/Acm1/Bmh1 ternary complex formation

Overview

The anaphase-promoting complex (APC) is an essential E3 ubiquitin ligase responsible for catalyzing proteolysis of key regulatory proteins in the cell cycle. Cdh1 is a co-activator of the APC aiding in the onset and maintenance of G_1 phase, while phosphorylation of Cdh1 at the end of G_1 phase by cyclin dependent kinases assists in the inactivation of APC^{Cdh1}. Here, we suggest additional components are involved in the inactivation of APC^{Cdh1} independent of Cdh1 phosphorylation. We have identified proteins known as Acm1 and Bmh1 that bind and form a ternary complex with Cdh1. The presence of phosphorylated Acm1 is critical for the ternary complex formation, and Acm1 is predominantly expressed in S phase when APC^{Cdh1} is inactive. The assembly of the ternary complex inhibits ubiquitination of Clb2 *in vitro* by blocking Cdh1's interaction with Clb2. *In vivo,* lethality caused by overexpression of constitutively active Cdh1 is rescued by overexpression of Acm1. Partially phosphorylated Cdh1 in the absence of *ACM1* still binds to and activates the APC. However, the addition of Acm1 decreases Clb2 ubiquitination when using either phosphorylated or nonphosphorylated Cdh1. Taken together, our results suggest an additional inactivation mechanism exists for APC^{Cdh1} that is independent of Cdh1 phosphorylation.

Introduction

The eukaryotic cell cycle is largely controlled by targeted proteolysis of regulatory proteins, specifically through ubiquitin-mediated proteolysis (1-4). The

anaphase-promoting complex (APC) is an essential E3 ubiquitin ligase responsible for catalyzing proteolysis of key regulatory proteins in the cell cycle. Substrate specificity of the APC is attributed to co-activator proteins that associate with particular substrates throughout the cell cycle (25,29,35). The co-activators Cdc20 and Cdh1 are thought to aid in the recruitment of particular substrates through substrate binding independent of the APC or in assemblies known as APC^{Cdc20} or APC^{Cdh1} (38,39,86). The APC poly-ubiquitinates substrates, marking them for degradation by the proteasome (32,35). Ubiquitin ligase activity of the APC has been shown to require the presence of Cdc20 or Cdh1 (27-29,32,33). Co-activators interact with the APC at specific times during the cell cycle, with Cdc20 activating the APC from early to late M phase and Cdh1 activating the APC from late M phase through G_1 (27,29,34).

Phosphorylation of Cdh1 has been demonstrated as an important control mechanism for the APC^{Cdh1} complex (57). Phosphorylation of Cdh1 is cell cycle dependent and is considered to be the main regulatory mechanism by which APC^{Cdh1} is inactivated. Cdh1 is phosphorylated in S phase, G_2, and mitosis and dephosphorylated in G_1 (57). The phosphorylation of Cdh1 corresponds to high levels of the cyclin-dependent kinase (CDK) activity. When the CDK consensus phosphorylation sites within Cdh1 from budding yeast were mutated to alanine, Cdh1 activated the APC constitutively, failing to accumulate mitotic cyclins (57). Cdh1 is phosphorylated at the G_1/S phase transition by G_1 and S phase cyclins that activate the CDK in budding yeast, Cdc28. Cdh1 remains hyperphosphorylated until late in mitosis when Cdc14 phosphatase is activated allowing Cdh1 to reactivate the APC (34,58). In S and M phases, Cdh1 failed to associate with the APC in Cdh1 co-immunoprecipitations (57). Additionally, *in vitro*

phosphorylation of recombinant Cdh1 by CDKs is sufficient for inactivation of the APC (87).

Protein-protein interactions have also been shown to be important in the regulation of Cdh1 (88,89). In higher eukaryotes, Emi1 and Mad2B have been shown to inhibit Cdh1 through protein-protein interactions (88,89). Although the function of Mad2B is unclear, Emi1 is reported to inhibit substrate binding to Cdh1. However, similar inhibitors in yeast have not yet been identified.

In order to discover potentially novel Cdh1 interacting proteins, we performed a proteomic screen of Cdh1 immunoprecipitations in budding yeast. In the process, we identified a multi-protein complex which forms with Cdh1, consisting of a previously uncharacterized protein Acm1 (YPL267W) and two members of the 14-3-3 protein family known as Bmh1 and Bmh2. The expression of Acm1 is cell cycle dependent, and Acm1 is necessary for complex formation with Cdh1 and Bmh1. Formation of the complex, which we refer to as the CAB complex or Cdh1/Acm1/Bmh1 complex, is restricted to the cell cycle window in which Acm1 is expressed. Without Acm1, Clb2 levels *in vivo* appear lower when compared to cells containing Acm1 under certain growth conditions. Complex formation inhibits APC^{Cdh1} activity *in vitro*, and lethality caused by overexpression of constitutively active Cdh1 is rescued by Acm1 overexpression. The CAB complex functions to block substrate binding with the co-activator. Partially phosphorylated Cdh1 binds and activates the APC in the absence of Acm1. Lastly, we have shown the ability of Acm1 and Bmh1 to inhibit APC^{Cdh1} despite the Cdh1 phosphorylation state. Taken together, our data suggests the existence of an additional inactivation mechanism of APC^{Cdh1} independent of Cdh1 phosphorylation.

Experimental Procedures

Cloning. Cloning for p415ADH-FLAGCdh1 and pNC219-FLAGCdh1m9 have been described (90). The resulting centromeric plasmid, p415ADH-FLAGCdh1, constitutively expresses 3xFLAG-*CDH1* from the *ADH* promoter. pNC219-FLAGCdh1m9 expresses the 3xFLAG-*CDH1* phosphomutant with 9 of 11 CDK sites mutated to alanine from the *GAL* promoter. The plasmids pHLP117 expressing 3HA-Acm1 from its natural promoter, pHLP107 expressing FLAG-Acm1 from the *ADH* promoter, and pHLP106 expressing FLAG-Acm1 from the *GAL1* promoter were gifts from M. Hall. The plasmids pRSETClb2, pET28-His_6-Cdh1, and pET28-His_6-Ubc4 were gifts from D. Barford (36). *BMH1* and *CLB2* were amplified by PCR from yeast genomic DNA and cloned into pGEX-4T-1 at the *BamH1* and *Xho1* sites yielding a GST tag on the amino terminus of Bmh1 and Clb2 for overexpression in *E. coli*. All PCR generated constructs were confirmed by DNA sequencing.

Strains and Media. Yeast strains are described in Table I. YPD (20g/L peptone [Fisher], 10g/L yeast extract [Bacto], 20g/L dextrose [MP Biomedicals]); YP (20 g/L peptone, 10 g/L yeast extract); synthetic medium (6.7 g/L yeast nitrogen base lacking amino acids [Difco], 20 g/L dextrose or 20 g/L raffinose, appropriate amino acid dropout mix [Q-Biogene]).

Cell Cycle Arrest. For G_1 arrest in *bar1* cells, α-factor peptide is added directly to the cultures during mid-log phase growth at 50 μg/L. *BAR1* cells require 3 μg/ml α-factor

peptide for an effective G_1 arrest. Arresting in S phase requires the addition of 10 mg/ml hydroxyurea (Sigma). M phase arrests are carried out by the addition of 15 mg/ml nocodazole (Sigma). This lab has acquired a *cdc15-2* yeast strain that allows cell cycle arrest in telophase induced by temperature shift from 25° C to 37° C. Cell cycle arrests are monitored by phase-contrast microscopy until >90% of the cells have achieved the desired morphology.

Cdh1 Purification. Yeast cells were grown asynchronously to OD_{600}=1 and lysed in 1 volume of APC-C lysis buffer (25mM HEPES-NaOH pH 7.5, 400mM NaCl, 10% glycerol, 0.1% Triton X-100, 0.1mM dithothreitol, 0.5mM PMSF, and complete protease inhibitor cocktail (Roche)) by vortexing for 45 min. at 4° C in 1.7 ml microcentrifuge tubes containing 0.5 ml 0.5 mm glass beads. Cell lysate was cleared by centrifugation at 16,000 x g for 15 min. Soluble extracts were pooled and cleared a second time at 5000 rpm for 5 min. Extracts were incubated with anti-FLAG M2 antibody-coupled resin (Sigma) for 1.5 hrs. at 4° C. Bound Cdh1 was washed extensively with APC-C buffer (25mM HEPES-NaOH pH 7.5, 400mM NaCl, 10% glycerol, 0.1% Triton X-100, 0.1mM dithothreitol, 0.5mM PMSF) and eluted 2X with FLAG peptide (250 µg/ml) in APC-C buffer.

APC Purification. Yeast cells were grown, lysed, and lysates cleared in the same manner as Cdh1. Extracts from YNL172W-TAP were incubated with Calmodulin affinity resin (Stratagene) for 1.5 hrs. at 4° C in APC-C buffer containing 1mM $CaCl_2$. 2 L of cells from YKA156 were grown to OD_{600}=0.5 at 25° C and temperature shifted to

37° C for 2 hours. Extracts from YKA156 were incubated with EZView anti-FLAG M2 antibody-coupled resin (Sigma) for 1.5 hrs. at 4° C, washed extensively with APC-C buffer and eluted 2X with FLAG peptide (250 μg/ml) in low salt APC-C buffer (25mM HEPES-NaOH pH 7.5, 150mM NaCl, 10% glycerol, 0.1% Triton X-100, 0.1mM dithothreitol).

Acm1 Purification. Yeast cells were grown, lysed, and lysates were cleared in the same manner as Cdh1 purifications. Extracts from YPL267W-TAP were incubated with Calmodulin affinity resin (Stratagene) for 1.5 hrs. at 4° C in APC-C buffer containing 1mM $CaCl_2$. Acm1 bound to beads were used in binding assays. Acm1 used in the ubiquitination assay and Clb2 binding assay was eluted with TAP elution buffer (40 mM Tris pH 7.5, 0.6mM DTT, 2.5 mM EDTA) for 30 minutes at room temperature while shaking at 700 rpm. $MgCl_2$ was added to the elution buffer at a final concentration of 15 mM. FLAG-Acm1 used in the ubiquitination assay and binding assays was purified in the same manner as APC.

Bmh1/Clb2 Purification. Overnight cultures of *E. coli* harboring *BMH1* or *CLB2* in the pGEX-4T-1 plasmid were grown and used to inoculate 1L of 2XYT (16g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl) containing 100 μg/ml ampicillin. Cultures were grown for 8 hours at 37° C, temperature shifted to 23° C and IPTG added (100 μM final). Cells were grown overnight and harvested by centrifugation. Cell pellet was resuspended in 1X PBS 0.5 M NaCl keeping suspension on ice. Cells were lysed 3X using sonication for 20 sec. intervals with a 40 sec. pause. Lysates were cleared by centrifugation at 4° C and

25,000 rpm for 30 min. Cleared lysates were incubated with 100 µl washed GSH beads (Amersham glutathione sepharose 4B) for 10 min. Beads and lysate were pipetted and transferred to Bio Rad micro columns. Beads were washed with 5 column volumes of 1X PBS 0.5 M NaCl. Bound protein was used on the GSH beads for binding assays. Bmh1 used in the ubiquitination assay was treated with biotinylated thrombin (Novagen) to cleave the GST tag. Sample was treated with streptavidin agarose to remove thrombin. Buffer was exchanged with 40 mM Tris pH 7.5, 0.6 mM DTT, 10 mM $MgCl_2$ using microcon filters with a 10 kDa cutoff (Millipore).

In Vitro Binding Assays. Compact reaction columns (USB) were used for all binding assays. Protein used as bait was immobilized on affinity beads and washed in the reaction column. Protein used as prey was added to each column and allowed to incubate for times noted at 4° C under rotation. Columns were washed with five column volumes of APC-C lysis buffer and eluted in 1X NuPAGE LDS sample buffer (Invitrogen) unless the immobilized protein used the TAP tag. In TAP purifications, APC-C buffer containing 1 mM $CaCl_2$ was used for washes. Five percent of each reaction was loaded on the gel.

Ubiquitination Assay. Ubiquitination assays were carried out as described by D. Barford (36). The substrate Clb2 (pRSETClb2), the co-activator Cdh1 (pET28-His_6-Cdh1), and the E2 Ubc4 (pET28-His_6-Ubc4) were prepared using the TNT T7 Quick coupled *in vitro* transcription/translation kit (Promega) unless otherwise noted. APC (YKA156) and Acm1 were purified from budding yeast (see APC and Acm1

purification) while Bmh1 was expressed and purified from *E. coli* (see Bmh1 purification). APC used in each reaction was 1/50 of the elution from 2 L of cells. Clb2 was radiolabeled with S^{35} methionine (Perkin Elmer). Reactions were run on a 4-12% Bis-Tris NuPAGE gel (Invitrogen), dried, and imaged using Hyperfilm (Amersham Biosciences).

Flow cytometry. Cells were prepared as described previously (91). DNA content was measured on a FACScan instrument and ModFit LT software (Verity Software House, Inc.) used to calculate percentages of G_1, S, and G_2/M cells present.

Protein Identification and Mass spectrometry. Elutions from Cdh1 purifications were acetone precipitated, reconstituted in 1X LDS sample buffer containing 100mM DTT and separated on a 4-12% Bis-Tris NuPAGE gel (Invitrogen). Gels were stained with Coomassie blue or silver. Protein bands were excised, destained and incubated with trypsin. After digestion, samples were frozen, lyophilized and resuspended in a small volume of 50% methanol/0.1% formic acid. Peptide masses were analyzed on a MALDI-TOF/TOF mass spectrometer (Applied Biosystems) and proteins identified using the MASCOT search engine (Matrix Science). Samples which needed further analysis were analyzed on a nano-ESI Q-TOF (Micromass) mass spectrometer.

Gel Filtration. FLAG immunoprecipitations were prepared from S phase arrested cells in APC-C buffer. The samples were fractionated on a Superdex 200 HR 10/30 gel filtration column with extraction buffer on an AKTA Explorer system (Amersham

Biosciences) at 0.4 ml/min. Fractions were collected in 0.5 ml increments and each fraction acetone precipitated. Proteins were resolved by SDS-PAGE and detected by immunoblotting with an α-FLAG antibody.

Results

Acm1 and Bmh1 associate with Cdh1 *in vivo* and *in vitro* and form a ternary complex. To identify potential interacting proteins of Cdh1, we used a targeted proteomic approach consisting of immunoprecipitations with genomically FLAG tagged Cdh1 followed by mass spectrometric analysis. We discovered three proteins known as Acm1, Bmh1, and Bmh2 in budding yeast which stably interact with Cdh1 under high salt wash conditions (Fig. 1.1A). The untagged parent strain was used as a control. C-terminal Cdh1 fragments were also identified by mass spectrometry. Interestingly, we did not identify any other specific proteins in the Cdh1 immunoprecipitation presumably due to the high salt wash conditions which disrupt all but the most stable protein complexes. Gel filtration analysis of the complex showed co-elution of Cdh1 and Acm1 in a stoichiometric interaction (data not shown). Acm1 (APC^{Cdh1} modulator 1) is a previously uncharacterized protein with no known function although it was originally identified as a potential CDK substrate (92). However, as members of the 14-3-3 family of proteins, Bmh1 and Bmh2 have been associated with a variety of cellular processes and have been defined as phosphoserine-binding proteins (93,94). We believe two possibilities for these interactions exist, either as potential APC^{Cdh1} substrates or as regulators of Cdh1.

To confirm the specificity of the interactions between Cdh1, Acm1, and Bmh1, we used *in vitro* binding assays. FLAG tagged Cdh1 and TAP tagged Acm1 were purified from yeast while recombinant Bmh1 was expressed in *E. coli*. GST-Bmh1 was immobilized on GSH beads and incubated with Cdh1-FLAG. GST bound to GSH beads was used as a control. Acm1-TAP was immobilized on calmodulin beads and incubated with Cdh1-FLAG. Empty calmodulin beads served as a control. Likewise, Acm1-TAP was incubated with GST-Bmh1. We observed a stable interaction between Bmh1/Cdh1, Acm1/Cdh1, and Acm1/Bmh1 under high-salt wash conditions (Fig. 1.1B). In order to see if any one component of the CAB complex was necessary for complex formation, we performed immunoprecipitations from strains deleted for components of the complex. We expressed N-terminally FLAG tagged Cdh1 in WT, *acm1Δ, bmh1Δ,* and *bmh2Δ* strains and immunopurified with anti-FLAG antibody followed by gel electrophoresis and mass spectrometric analysis. Gel slices for each region where Cdh1, Acm1, Bmh1, and Bmh2 migrate were analyzed. Bmh1 and Bmh2 cannot be detected in the absence of Acm1. In the *bmh1Δ* strain, Acm1 and Bmh2 still associated with Cdh1. Likewise, Acm1 and Bmh1 still associated with Cdh1 in the *bmh2Δ* strain (Fig**.** 1.1C). Additionally, we incubated purified FLAG-Cdh1 from either wild type or *acm1Δ* cells with immobilized GST-Bmh1 in an *in vitro* binding assay. Without Acm1, Cdh1 does not bind Bmh1 (Fig. 1.1D, lane 6)**.** The Bmh1/Cdh1 binding in Fig. 1.1B is attributed to the presence of Acm1 which co-purifies with Cdh1. Together these findings illustrate the dependence of the CAB complex formation on Acm1 as well as a possible redundancy between Bmh1 and Bmh2.

Acm1 is expressed in S phase and has an effect on cell cycle progression. Because Cdh1 activates the APC in a cell cycle dependent manner, the interaction with Acm1 and the 14-3-3 proteins could also fluctuate throughout the cell cycle. In order to determine whether these proteins were regulated in a cell cycle dependent manner, Acm1-TAP and Bmh1-TAP protein levels were analyzed in cell lysates from G_1, S, and M phase arrested cells. Acm1 levels were found to be significantly higher in S phase over the other cell cycle phases, while Bmh1 levels remain constant in each cell cycle stage (Fig. 1.2A). This finding is in agreement with the Acm1 mRNA expression pattern throughout the cell cycle (95). Analysis of Acm1 mRNA expression levels from the *Saccharomyces* Genome Database revealed the closest pattern of gene expression to Acm1 is observed for Clb5 (95). Clb5-Cdc28 is believed to be the primary cyclin-CDK complex responsible for phosphorylation of Cdh1 during S phase, although other G_1 cyclin-CDK complexes are also involved (34,96,97). Acm1 protein levels are highest in S phase when Cdh1 is phosphorylated and APC^{Cdh1} becomes inactivated. This observation suggested an involvement of Acm1 in phosphorylation-mediated regulation of APC^{Cdh1} during S phase.

We did not expect the deletion of *ACM1* to induce large perturbations in the cell cycle. According to the *Saccharomyces* Genome Database, cells with *ACM1* systematically deleted were viable (98). Indeed, *acm1Δ* cells grow at normal rate when compared to WT cells. In order to see if deleting *ACM1* had any effect within a single cell cycle, cells from *acm1Δ* and wild type strains were synchronized in S phase with hydroxyurea, released and analyzed by flow cytometry for DNA content in 20 minute intervals (Fig. 1.2B). Interestingly, FACS analysis of *acm1Δ* cells showed an increase

(14%) in the S phase cell population in comparison to wild type cells at 80 minutes after release. This effect was reproducible but to slightly varying degrees (data not shown). Additionally, the *acm1Δ* strain resynchronized with WT cells in M phase. The effect in S phase progression in *acm1Δ* cells suggests Acm1 may play a role in cell cycle progression throughout S phase. If the CAB complex does play a role in APC^{Cdh1} regulation, one would expect a difference in APC^{Cdh1} substrate levels in cells lacking Acm1. In order to test whether APC^{Cdh1} substrates are affected in *acm1Δ* cells, we performed a G_1 block-release experiment in cells containing Acm1 compared to cells void of Acm1 and examined levels of the known APC^{Cdh1} substrate Clb2. Interestingly, we found a slight decrease in Clb2 levels in early time points in the absence of Acm1 under certain growth conditions (Fig. 1.2C). The cycling pattern of Clb2 corresponds to that of Acm1 although Acm1 increases to its maximum slightly before Clb2 reaches its highest level. An increased level of APC^{Cdh1} activity in the strain lacking Acm1 could explain this difference in Clb2 levels between the two strains. However, the effects in the cell cycle are slight, so CAB complex formation may exist as an overlapping inhibitory mechanism in addition to phosphorylation for APC^{Cdh1}.

CAB complex formation inhibits APC^{Cdh1} mediated ubiquitination of Clb2. Since Acm1 was predominantly expressed in S phase when APC^{Cdh1} activity is inhibited and cells lacking Acm1 exhibited a difference in Clb2 levels, we investigated whether the CAB complex formation affected APC^{Cdh1} activity directly. To demonstrate a possible inhibitory effect of Acm1 and Bmh1 on APC activity, an *in vitro* ubiquitination assay was used to measure ubiquitination of the substrate Clb2 (Fig. 1.3A). APC and TAP

tagged Acm1 were purified from yeast while Ubc4 (E2), Cdh1, and S^{35}-labeled Clb2 were expressed *in vitro* and incubated together for the assay. The degree of substrate ubiquitination was measured by phosphorimage analysis of S^{35}-labeled Clb2. With Bmh1 levels held constant, increasing concentrations of Acm1-TAP resulted in a dose-dependent inhibition of Clb2 ubiquitination (lanes 3, 4, and 5). A mock purification from *acm1Δ* cells was used to rule out buffer or non-specific protein effects on APC^{Cdh1} activity and illustrates Clb2 ubiquitination levels without any inhibition (lane 6). In order to quantify Acm1 inhibition relative to APC and Cdh1 levels, we used yeast purified FLAG-tagged APC, Cdh1, and Acm1 (Fig. 1.3B). Acm1 appears to inhibit APC activity at substoichiometric levels (Fig. 1.3B, lanes 5-7 and 10-12) although we have observed stoichiometric binding from our gel filtration data. We attribute this result to the presence of a certain percentage of inactive Cdh1 in the preparation. No inhibitory effect on Clb2 ubiquitination was observed upon the addition of Bmh1 alone (Fig. 1.3B, lane 4). This is not surprising due to the requirement of Acm1 for the Bmh1/Cdh1 interaction. From our *in vitro* ubiquitination assay results, we conclude Acm1 and Bmh1 have an inhibitory effect on APC^{Cdh1} ubiquitin ligase activity.

To investigate whether Acm1 inhibits APC^{Cdh1} activity *in vivo*, we co-expressed Acm1 and the constitutively active Cdh1 mutant containing 9 of the 11 CDK phosphorylation sites mutated to alanine. It has been previously shown that Cdh1 phosphomutants mimicking non-phosphorylated Cdh1 were unable to accumulate Clb2 and Clb3, to form mitotic spindles, and undergo cytokinesis (57). We found overexpression of Acm1 rescues the lethality caused by overexpression of the Cdh1 phosphomutant (Fig. 1.3C). From this experiment, we concluded the CAB complex

formation suppresses APC^{Cdh1} activity as an overlapping regulatory mechanism in the absence of complete CDK phosphorylation. This mechanism exists in addition to Cdh1 phosphorylation which has clearly been shown to also inhibit APC^{Cdh1} activity. CAB complex formation may function then to inhibit APC^{Cdh1} regardless of the phosphorylation status of Cdh1.

Our next question was how CAB complex formation inhibited APC^{Cdh1} activity. Enzymatic activity of APC^{Cdh1} is mediated by the interaction between Cdh1, APC, and the substrate. Therefore, we hypothesized the complex either blocked APC binding to Cdh1 or blocked Cdh1 binding to Clb2. To determine whether Acm1 and Bmh1 were inhibiting APC^{Cdh1} activity by blocking the APC/Cdh1 interaction or by blocking substrate binding to Cdh1, we performed binding assays between Cdh1-APC and Cdh1-Clb2 with or without the presence of the other complex components (Fig. 1.3D, 1.3E). Bacterially expressed GST-Clb2 was incubated with FLAG-Cdh1 from *acm1Δ* cells after Cdh1 was pre-incubated with varying levels of Acm1 in an *in vitro* binding assay. In the second *in vitro* binding assay, N-terminally tagged FLAG-Cdh1 was purified from WT and *acm1Δ* cells and incubated with APC via Apc1-TAP. Although, no difference in Cdh1 binding to the APC could be seen in the presence or absence of Acm1 (Fig. 1.3E, compare lane 13 and 17), Cdh1 binding to Clb2 decreased in a dose dependent manner with increasing levels of Acm1 (Fig. 1.3D, lanes 5-8). This observation suggests Acm1 disrupts the interaction between the substrate and Cdh1 which may explain how CAB complex formation inhibits APC^{Cdh1} activity.

Formation of the complex is phosphorylation dependent. Current data supports the important role Cdh1 phosphorylation plays in the inactivation of APC^{Cdh1} activity. Therefore, we hypothesized phosphorylation had some involvement with the interaction between Cdh1, Acm1, and Bmh1. To determine what role phosphorylation plays in the complex, we employed an *in vitro* binding assay between Bmh1 and Cdh1 with and without phosphatase treatment of Cdh1 (Fig. 1.4A). Cdh1-FLAG was purified from WT cells, treated with λ-phosphatase, and incubated with immobilized GST-Bmh1 in an *in vitro* binding assay. Cdh1-FLAG treated with phosphatase buffer but without λ-phosphatase was used as a control. A shift in Cdh1 due to dephosphorylation can be seen when run on a 7% Tris-Acetate gel (Fig. 1.4A lower panel). Upon phosphatase treatment, Cdh1 no longer interacted with Bmh1 (compare lane 6 and 9). However, since Cdh1 was purified from WT cells, Acm1 is present. As shown previously, Acm1 is needed for the Cdh1/Bmh1 interaction. Acm1 contains 5 copies of the consensus CDK recognition sequence (S/T-P-x-K/R), and we have observed that Acm1 is heavily phosphorylated in normal Cdh1 purifications from asynchronous cells. From this assay we could not conclude whether the dephosphorylation of Cdh1, Acm1, or both that result in the diminished binding of Cdh1 with Bmh1. To determine whether phosphorylation of Cdh1 or Acm1 contribute to formation of the complex, we purified N-terminally tagged FLAG-Cdh1 from *acm1Δ* cells and N-terminally tagged HA-Acm1 from *cdh1Δ* cells, and treated each with phosphatase in an *in vitro* binding assay. Surprisingly, we found that phosphorylation is not needed for the interaction between Cdh1 and Acm1 and the addition of Bmh1 had little effect (Fig. 1.4B). To determine whether Acm1 phosphorylation affects Bmh1 binding, an *in vitro* binding assay was employed with

immobilized GST-Bmh1 incubated with phosphatase treated or untreated FLAG-Acm1 from *cdh1Δ* cells. Dephosphorylation of Acm1 abolished binding to Bmh1 indicating that phosphorylation of Acm1 but not Cdh1 is critical for the CAB complex formation (Fig. 1.4C**,** lanes 4 and 8).

Phosphorylated Cdh1 binds to and activates the APC *in vitro* and is inhibited by complex formation. Knowing Acm1 has the ability to rescue lethality caused by expressing constitutively active Cdh1 with 9 of the 11 CDK sites mutated to alanine, we hypothesized partially phosphorylated Cdh1 could still bind and activate the APC. First, we investigated whether *in vivo* phosphorylated Cdh1 could still bind to the APC in the absence of the complex. To test this notion, we bound Cdh1 to the APC in an *in vitro* binding assay and then used phosphatase treatment on the complex to illustrate all bound Cdh1 to the APC is still partially phosphorylated. N-terminally tagged FLAG-Cdh1 was purified from asynchronous *acm1Δ* cells and incubated with yeast purified APC via Apc1-TAP for 30 min. at 4° C. After incubation with Cdh1, the immobilized APC/bound Cdh1 was washed extensively, divided into two columns, and one column treated with λ-phosphatase. After treatment, samples were separated by SDS-PAGE and analyzed by Western blot. We found all Cdh1 bound to the APC was phosphorylated. Samples were run on a 6% Tris Glycine gel to differentiate phosphorylated from dephosphorylated Cdh1. A clear mobility shift due to dephosphorylation of bound Cdh1 can be seen from untreated and phosphatase treated samples (Fig. 1.5A). We believe the Cdh1 bound to the APC is only partially phosphorylated due to the existing evidence that fully phosphorylated Cdh1 cannot bind or activate the APC (57,87). This suggests partial *in*

vivo phosphorylation of Cdh1 in the absence of the complex does not fully inhibit the interaction between Cdh1 and the APC.

Since partial phosphorylation of Cdh1 did not disrupt the interaction with the APC, we investigated whether the phosphorylated coactivator could activate the APC. From the previous experiments, we knew Cdh1 phosphorylation was not a prerequisite for the interaction with Acm1. So, we also examined whether the addition of Acm1 and Bmh1 could attenuate Clb2 ubiquitination using phosphorylated or nonphosphorylated Cdh1. In order to test this, we purified Cdh1 from *acm1Δ* cells, divided the sample for dephosphorylation by phosphatase, and used phosphorylated and dephosphorylated Cdh1 in the *in vitro* ubiquitination assay (Fig. 1.5B). Cdh1 was washed extensively to remove phosphatase prior to adding Cdh1 to the assay. We added equivalent amounts of Cdh1 to both sets of reactions. Both partially phosphorylated and dephosphorylated Cdh1 were sufficient for APC mediated ubiquitin ligase activity (lanes 3 and 8). Upon the addition of Acm1 and Bmh1, a decrease in Clb2 ubiquitination can be seen using λ-phosphatase treated and untreated Cdh1 (lanes 4, 5, 9, and 10). Although Cdh1 phosphorylation is not necessary for Acm1 binding and inhibition, Acm1 and Bmh1 may exist as an early inhibition mechanism of partially phosphorylated Cdh1 before the cyclin dependent kinase can fully phosphorylate Cdh1 for inactivation. Acm1 and Bmh1 may exist as an overlapping inhibitory mechanism regardless of the phosphorylation status of Cdh1 to ensure proper APC inactivation at the G_1/S phase transition and during S phase.

Discussion

Previous work has shown that the phosphorylation of Cdh1 occurs at the end of G_1 phase by the cyclin dependent kinase Cdc28 and has demonstrated that phosphorylation is responsible for the inactivation of the APC^{Cdh1} complex (57,87,99). Non-phosphorylatable Cdh1 mutants constitutively activate the APC prohibiting cells from accumulating Clb2 and Clb3 (57). Additionally, *in vitro* phosphorylation of recombinant Cdh1 is convincingly sufficient for inactivation of the APC (87). There is no question phosphorylation of Cdh1 plays an important role in APC inactivation. Because the cell cycle follows a very strict schedule, proper and timely inactivation of the APC must occur. Because Cdh1 has been shown to bind substrates independent of the APC (33,37,38), one reason for the strict regulation of Cdh1 may be to prevent the co-activator from interfering with substrates during parts of the cell cycle where it is not needed. It is logical then that cells have overlapping inactivation mechanisms to be sure certain cellular processes are disabled. The possibility remains for novel proteins to exist for APC^{Cdh1} inactivation. Here, we have identified an additional mechanism that acts independently of Cdh1 phosphorylation to efficiently inhibit Cdh1 from activating the APC. The mechanism is comprised of complex formation of Acm1 and Bmh1 with Cdh1 (CAB complex) and functions to inhibit APC^{Cdh1} activity by blocking substrate binding to the co-activator. The CAB complex is the first APC^{Cdh1} inhibitor identified in yeast. Acm1 is the critical component involved in complex formation and is cell cycle regulated. Hence, the Cdh1/Bmh1 interaction is confined to the cell cycle interval during which Acm1 is expressed.

We identified Acm1, Bmh1, and Bmh2 as Cdh1 interacting proteins using Cdh1 immunoprecipitations. *BMH1* and *BMH2* appear to be functionally redundant and while

deletion of both is lethal in most strains, cells remain viable with the deletion of only one of the genes (100,101). Bmh1 and Bmh2 are members of the 14-3-3 class of proteins whose roles include signal transduction, checkpoint control, apoptotic, and nutrient-sensing pathways, as well as subcellular localization of binding partners (94,102). 14-3-3 proteins have been defined as phosphoserine-binding proteins with ligands typically containing two different binding motifs, RSXpSXP or RXY/FXpSXP, although variations of these motifs exist (93,103). The third member of the complex that forms with Cdh1 is a previously uncharacterized protein named Acm1. Acm1 has been shown to interact with Bmh2 via yeast two hybrid assays (104). Acm1 has been identified as a potential Cdc28 substrate and contains five potential CDK phosphorylation sites which match the consensus recognition sequence S/T-P-x-K/R (92). Interestingly, the gene expression pattern of Acm1 is similar to that of Clb5, whose accumulation is necessary for Cdh1 phosphorylation and S phase entry. Although we have not seen any significant sequence homology of Acm1 in mammalian species, with such an important function, the possibility remains an analog exists in higher eukaryotes.

Inhibition of Cdh1 must occur in a timely manner for S phase to proceed. Acm1's presence is therefore necessary at the specific time when APC^{Cdh1} needs to be inactivated. Indeed, protein levels of Acm1 are highest in S phase coinciding with the phosphorylation of Cdh1 by Clb5-Cdc28. Deletion of Acm1 causes a perturbation in the cell cycle leading to an increased population of cells in S phase. Currently, we cannot decipher whether the increased S phase population is a result of early entry into S phase or slower progression through S phase. Additionally, we have observed slightly lower Clb2 levels in *acm1Δ* cells after a G_1 arrest and release suggesting an increased APC^{Cdh1}

activity in the absence of Acm1. The lower levels of Clb2 occurred in early time points under certain growth conditions. However, Clb2 levels return to a comparable level at later time points in the same experiment, which is attributed to effective inhibition of APC^{Cdh1} by phosphorylation. The cell cycle perturbation may not be explained by differing Clb2 levels but a longer S phase as a result of lower CDK activity could occur (105). However, our data can only suggest Acm1 has some regulatory role during S phase. Further experiments are needed to determine whether levels of S phase cyclins differ in *acm1Δ* cells.

Phosphorylation of Cdh1 occurs in S phase and complete phosphorylation disrupts the binding interface between Cdh1 and the APC. We have shown *in vivo* phosphorylated Cdh1 binds to and activates the APC although this result can be explained if Cdh1 is only partially phosphorylated. We have demonstrated Acm1 and Bmh1 have a direct inhibitory effect on APC^{Cdh1} activity *in vitro* and *in vivo* and that inhibition by Acm1 and Bmh1 can occur when Cdh1 is phosphorylated or dephosphorylated. Although the phosphorylation status of Cdh1 does not affect CAB complex formation, phosphorylation of Acm1 plays a critical role in the assembly of the ternary complex with Bmh1 and Cdh1. We have identified several phosphopeptides within Acm1 from Cdh1 immunoprecipitations. This is not surprising because Acm1 was originally identified as a potential Cdc28 substrate (92). Dephosphorylation of purified Acm1 has no effect on Cdh1 binding but does abolish Bmh1 binding. Phosphorylation of Acm1 and Cdh1 may occur in parallel by the same kinase to mediate complex formation. We have also observed a slight shift in the gel mobility of Acm1 before it is degraded. The gel shift could be a result of dephosphorylation perhaps

playing a role in Acm1 stability. Since Bmh1 binds only phosphorylated Acm1 and has no inhibitory role by itself, it may serve to protect Acm1 from early degradation. Whether or not phosphorylation of Acm1 or if the 14-3-3 proteins are required for APC^{Cdh1} inhibition is addressed in the next chapter.

Potentially, Acm1 and the 14-3-3 proteins are APC substrates. Protein levels of Acm1 drop sharply after S phase when the APC is reactivated. Although *ACM1* mRNA expression drops after S phase, the residual Acm1 left over may need to be removed to prevent premature inhibition of APC^{Cdh1}. Proteasomal degradation mediated by APC^{Cdc20} would make sense in order to remove Acm1 until it is needed for APC^{Cdh1} inactivation. The destruction box (D box) and the KEN box have been identified as degradation motifs in APC substrates (33) while Acm1 contains two potential D boxes (RTIL at aa8 and RIAL at aa 119) and a potential KEN box (KENLS at aa 98). Unlike APC substrates, Bmh1 levels are abundant throughout the cell cycle and its levels do not fluctuate. Additionally, Bmh1 does not interact with Cdh1 without Acm1 present. For these reasons it is unlikely Bmh1 is an APC substrate. Further examination of Acm1 as an APC substrate is addressed in the next chapter.

Interestingly, there appear to be some similarities between the APC^{Cdh1} inhibitor, Emi1, in higher eukaryotes and the CAB complex in yeast. Both inhibit APC^{Cdh1} activity *in vitro* and directly bind Cdh1 (62). Emi1 and Acm1 are both present during S phase and are degraded during M phase (69). In a recent report, a model system has been proposed where Emi1 acts as a pseudosubstrate inhibitor of APC^{Cdh1} and competes with other D-box containing substrates for Cdh1 binding (63). Additionally, we have observed an interaction between Acm1 and C-terminal fragments of Cdh1 in gel filtration

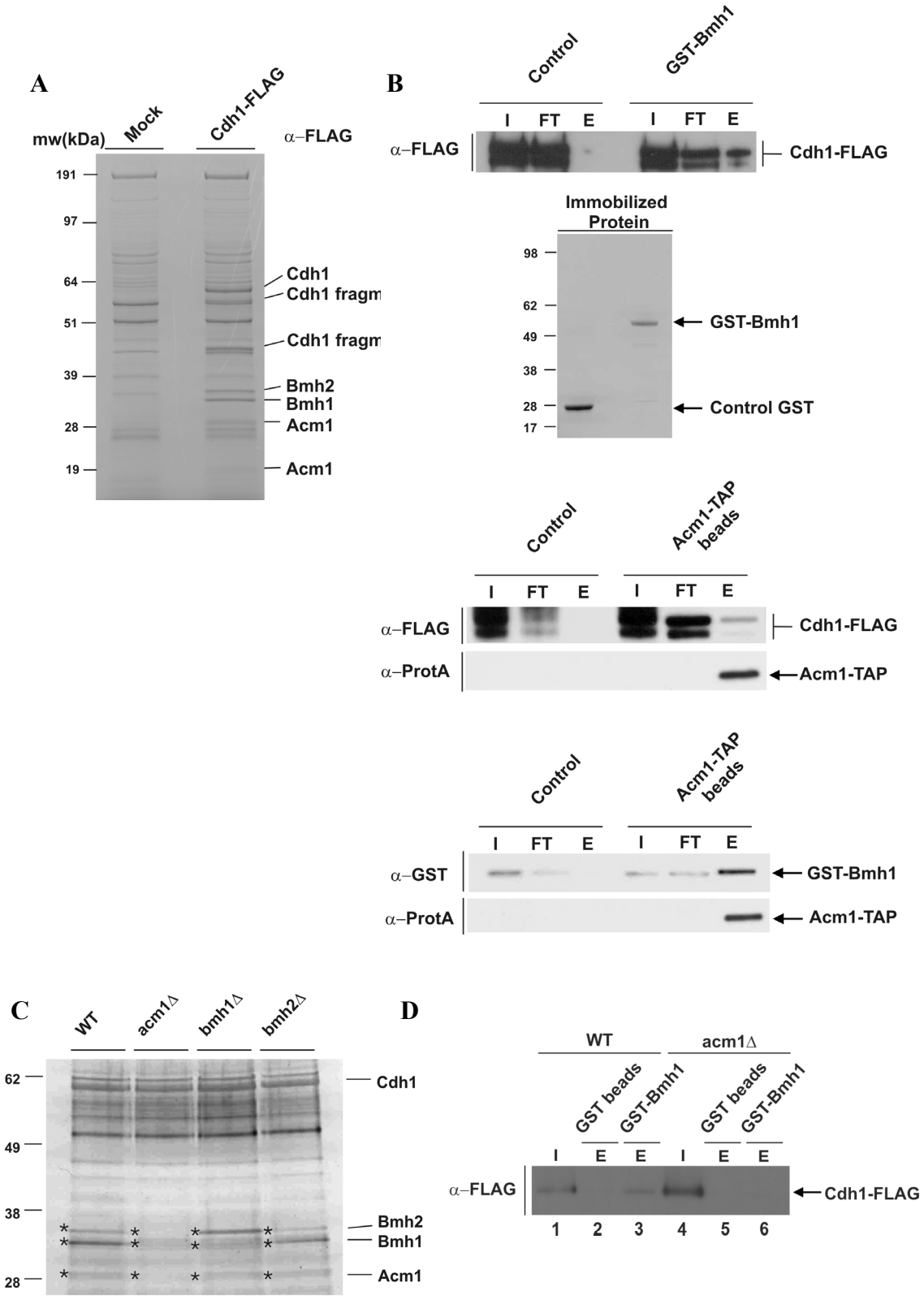
experiments. The C-terminus of Cdh1 houses a WD-40 domain shown to be the site for substrate binding (38). The mechanism of inhibition including the regions of Acm1 involved in Cdh1 binding and whether or not Acm1 mimics a substrate to inhibit APC^{Cdh1} activity will be the focus of the next chapter.

Cdh1 has been shown to relocalize from the nucleus to the cytoplasm removing it from the vicinity of the APC after phosphorylation presumably as a redundant inhibitory mechanism (73). 14-3-3 proteins are involved in the relocalization of the cell cycle regulatory protein Cdc25, so feasibly Acm1 and Bmh1 could play a similar role with Cdh1 (106,107). However, the possibility of Acm1 and Bmh1 affecting Cdh1 localization has not been thoroughly investigated.

Conceivably, additional mechanisms exist for APC inactivation through other protein interactions (65,71). Our evidence suggests that the complex formation serves as an overlapping mechanism in addition to the phosphorylation message to efficiently inhibit APC^{Cdh1} activity prior to entering mitosis (Fig. 1.6). Additional studies to further elucidate the biological significance and mechanism by which CAB complex formation inhibits APC^{Cdh1} are illustrated in the next chapter.

Table 1
S. cerevisiae strains used in Chapter 2

Strain	Relevant Genotype	Source
W1588-4c	*MAT*a *ade2-1 can1-100 His3-11 15 leu2-3 112 trp1-1 ura3-1*	R. Rothstein
YKA150	W1588-4c *bar1Δ::URA3*	Hall et al. 2004
YKA154	YKA150 *CDH1-3FLAG*::KanMX4	This Study
YKA323	YKA150 *ypl267wΔ*::KanMX4	This Study
DLY3033	*MAT*a *bar1::URA3 cdc15-2 ura3 leu2 trp1*	J. Pringle
YKA156	DLY3033 *CDC27-3FLAG*::KanMX4	This Study
BY4741	*MAT*a *his3-D1 leu2-D0 met15-D0 ura3-D0*	Open Biosystems
YPL267W-TAP	BY4741 *Acm1-TAP*::HIS3MX6	Open Biosystems
YKA322	YPL267W-TAP *cdh1Δ*::KanMX4	This Study
YER177W-TAP	BY4741 *Bmh1 -TAP*::HIS3MX6	Open Biosystems
YNL172W-TAP	BY4741 *APC1-TAP*::HIS3MX6	Open Biosystems
YGL003CΔ	BY4741 *cdh1Δ*::KanMX4	Open Biosystems
YPL267WΔ	BY4741 *acm1Δ*::KanMX4	Open Biosystems
YER177WΔ	BY4741 *bmh1Δ*::KanMX4	Open Biosystems
YDR099WΔ	BY4741 *bmh2Δ*::KanMX4	Open Biosystems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mw(kDa)
Mock
Cdh1-FLAG
α–FLAG
191
97
64
51
39
28
19
Cdh1
Cdh1 fragm
Cdh1 fragm
Bmh2
Bmh1
Acm1
Acm1
B
Control
GST-Bmh1
I FT E I FT E
α–FLAG
Cdh1-FLAG
Immobilized Protein
98
62
49
38
28
17
GST-Bmh1
Control GST
Control
Acm1-TAP beads
I FT E I FT E
α–FLAG
Cdh1-FLAG
α–ProtA
Acm1-TAP
Control
Acm1-TAP beads
I FT E I FT E
α–GST
GST-Bmh1
α–ProtA
Acm1-TAP
C
WT
acm1Δ
bmh1Δ
bmh2Δ
62
49
38
28
Cdh1
Bmh2
Bmh1
Acm1
D
WT
acm1Δ
GST beads
GST-Bmh1
GST beads
GST-Bmh1
I E E I E E
α–FLAG
Cdh1-FLAG
1 2 3 4 5 6

FIGURE 1.1

Figure 1.1: Acm1, Bmh1, and Cdh1 form a ternary complex.

A, Immunoprecipitations were performed with the yeast strain YKA154 containing Cdh1 with a 3xFLAG epitope at the C-terminus. The untagged parent strain was used as a control. Four liters of cells were grown to mid-log phase and arrested in G_1 with α-factor. Once harvested, cells were lysed and subjected to antibody affinity pulldowns using 100 µl of the α-FLAG antibody beads. Samples were run on 4-12% Bis-Tris gels (Invitrogen) and Coomassie stained. From each lane, gel slices were excised throughout the entire length of the gel. Proteins in each gel slice were digested with trypsin, analyzed by mass spectrometry, and identified through subsequent database searching. Those proteins present in the sample lane and absent in the control lane were considered to interact with Cdh1. Three proteins known as Bmh1, Bmh2, and a previously uncharacterized protein, Acm1, were identified as Cdh1 interacting proteins. **B,** GST-Bmh1 from *E. coli* immobilized on 10 µl GSH beads or Acm1-TAP from 1 L of asynchronous yeast cells (OD_{600}=1) immobilized on 60 µl calmodulin affinity beads were used in the compact reaction columns for the respective binding assay. 10 µl of purified Cdh1-FLAG from 1 L of asynchronous yeast cells or 10 µl of purified GST-Bmh1 were incubated with the immobilized protein for 2 hours at 4° C and washed extensively with high salt buffer. Bound proteins were eluted in 1X LDS buffer, run on 4-12% Bis Tris gels and transferred to membranes for Western analysis. I, FT, and E designate input, flow through, and elution. GST immobilized on GSH beads or empty calmodulin beads were used as controls. Immobilized protein shown is Coomassie stained and is 20X greater than that shown in binding assay. Cdh1 bound to Bmh1 and Acm1, and Bmh1 bound to Acm1. **C,** Cdh1, harboring an N-terminal 3xFLAG epitope transcribed from the *ADH* promoter on a centromeric plasmid, was expressed by transforming the plasmid into WT, *acm1Δ, bmh1Δ,* and *bmh2Δ* strains. Each strain was grown asynchronously in 1 L of selective media to OD_{600}=1 before harvesting and pulling down Cdh1 with 25 µl anti-FLAG M2 antibody-coupled resin (Sigma). Proteins were separated on a 4-12% Bis-Tris gel and Coomassie stained. *Gel slices from each lane were excised in the region for which each protein migrates, digested with trypsin, and analyzed by mass spectrometry in order to detect the presence of peptides corresponding to the proteins of interest. Neither Bmh1 nor Bmh2 could be detected in the *acm1Δ* strain. Bmh1 peptides could not be detected in *bmh1Δ*, but Acm1 and Bmh2 were present. Likewise, Bmh2 could not be detected in *bmh2Δ*, but Acm1 and Bmh1 were present. **D,** Cdh1 was purified from 2 L of asynchronous cells grown to OD_{600}=1 from either WT or *acm1Δ* strains using 50 µl α-FLAG antibody beads . GST-Bmh1 was immobilized on 5 µl of GSH beads in compact reaction columns. 2 µl of FLAG-Cdh1 purified from WT and *acm1Δ* cells was incubated with the immobilized protein for 2 hours at 4° C and washed extensively with high salt buffer. Bound proteins are eluted in 1X LDS buffer, run on 4-12% Bis Tris gels and transferred to membranes for Western analysis. An α-FLAG antibody was used to detect the presence of Cdh1. The absence of Acm1 abolishes the Bmh1/Cdh1 interaction.

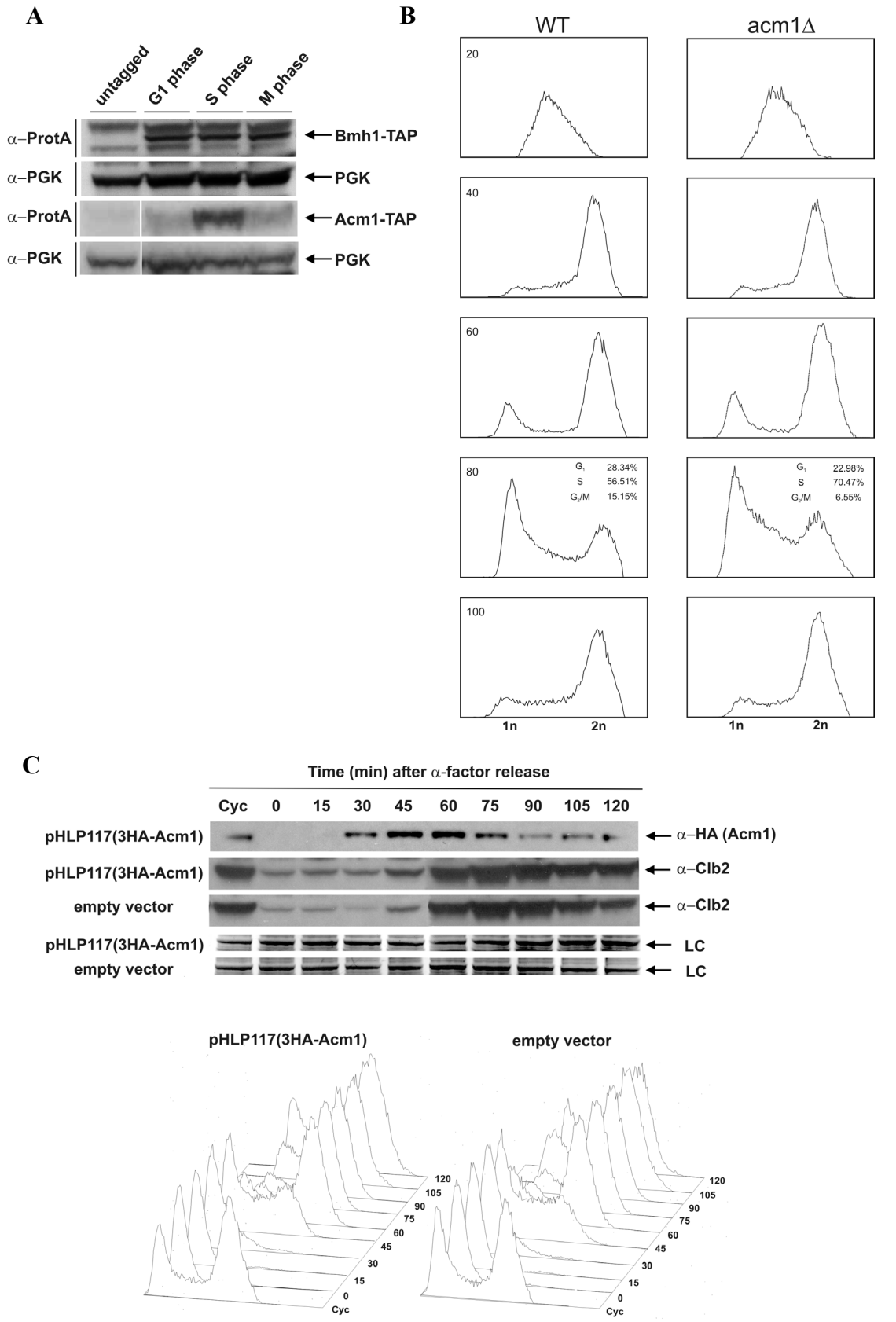

A
untagged
G1 phase
S phase
M phase
α–ProtA
Bmh1-TAP
α–PGK
PGK
α–ProtA
Acm1-TAP
α–PGK
PGK
B
WT
acm1Δ
20
40
60
80
100
G1 28.34%
S 56.51%
G2/M 15.15%
G1 22.98%
S 70.47%
G2/M 6.55%
1n
2n
1n
2n
C
Time (min) after α-factor release
Cyc 0 15 30 45 60 75 90 105 120
pHLP117(3HA-Acm1)
α–HA (Acm1)
pHLP117(3HA-Acm1)
α–Clb2
empty vector
α–Clb2
pHLP117(3HA-Acm1)
LC
empty vector
LC
pHLP117(3HA-Acm1)
empty vector
120
105
90
75
60
45
30
15
0
Cyc

FIGURE 1.2

Figure 1.2: Acm1 is expressed in S phase and the deletion of Acm1 leads to an enriched S-phase cell population. A, Strains containing either Acm1 or Bmh1 with a C-terminal TAP genomic tag were grown to mid-log phase and arrested in G_1 with α-factor, S phase with hydroxyurea, and M phase with nocodazole. Cells were harvested, lysed and extracts run on 4-12% Bis-Tris gels. Western analysis using an anti-Protein A antibody was performed to measure protein levels in each cell cycle phase. Acm1 levels are highest in S phase while Bmh1 levels remain constant throughout the cell cycle. **B,** Cells from WT and *acm1Δ* strains were arrested with hydroxurea for synchronization and released into fresh media. Samples were collected in 20 minute intervals for 100 minutes after release. DNA was stained using SYTOX Green (Molecular Probes) and DNA content measured on a FACScan instrument for cell cycle progression. Time points for the FACS analysis are present from 20 minutes to 100 minutes. An enriched S phase population was observed at 80 minutes in the *acm1Δ* strain when compared to WT cells. Percentages of G_1, S, and G_2/M cells for WT and *acm1Δ* strains at 80 minutes are shown. ModFit LT software (Verity Software House, Inc.) was used to calculate percentages of G_1, S, and G_2/M cells. **C**, Cells from the *acm1Δ* strain (YKA323) containing the plasmid pHLP117 expressing HA-Acm1 from its natural promoter or cells from the same strain containing the empty plasmid were grown to mid-log phase and arrested in G_1 with α-factor. Cells were released from the G_1 arrest by washing with 4x the culture volume (fresh media) and resuspending washed cells in fresh media. Time points were taken in 15 minute intervals. 40 μg of extract was loaded on the gels and probed with an α-HA antibody or an α-Clb2 antibody. The Coomassie stained membranes were used as loading controls. A decrease in Clb2 levels is seen from timepoints 0-45 min. FACS analysis was performed for each 15 minute time point of the G_1 arrest and release.

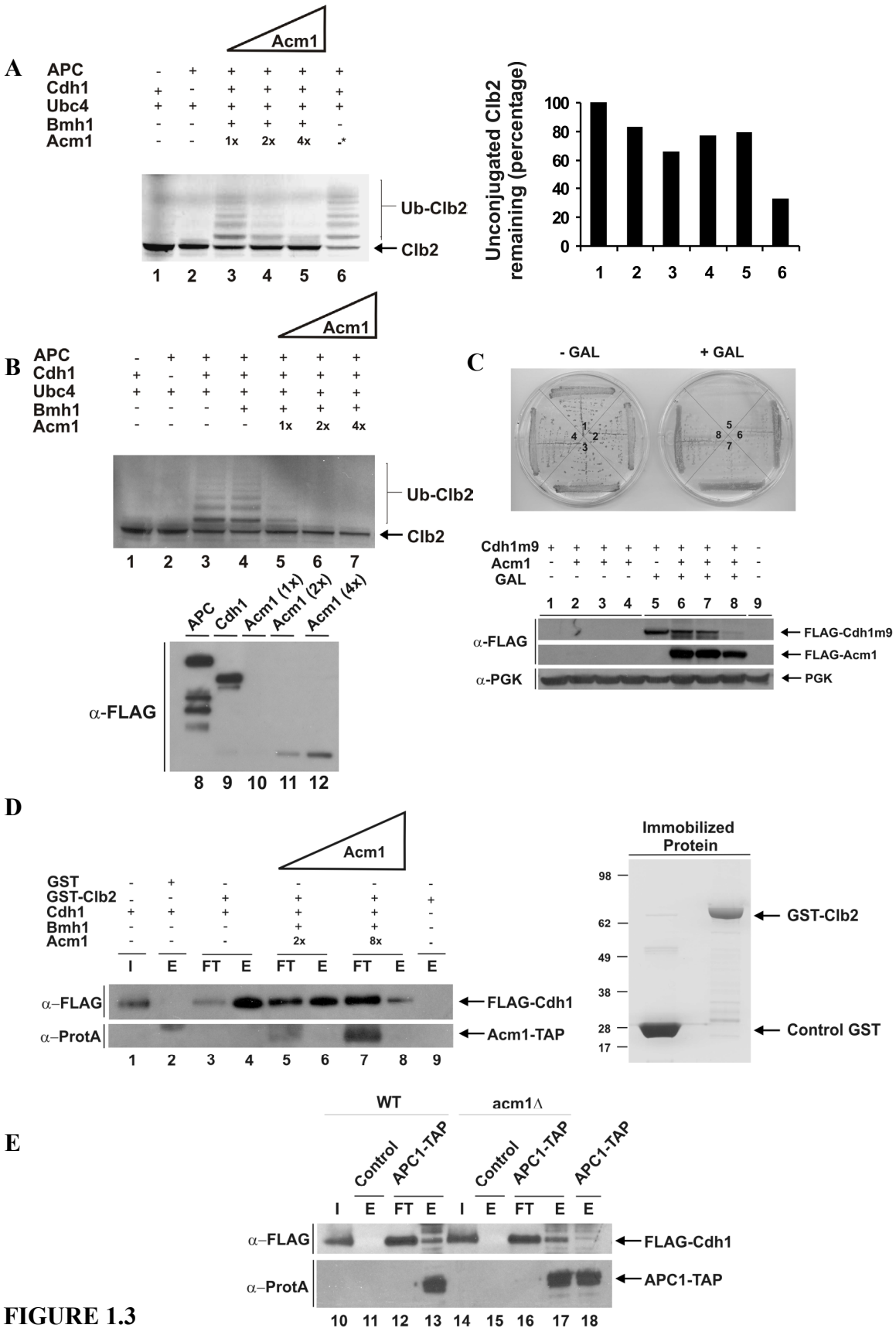
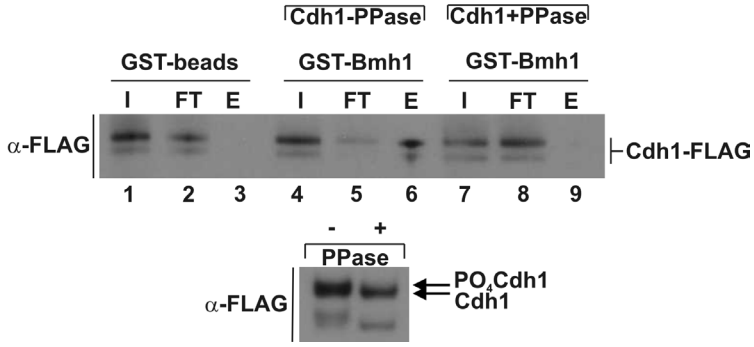

A
Acm1
APC - + + + + +
Cdh1 + - + + + +
Ubc4 + + + + + +
Bmh1 - - + + + -
Acm1 - - 1x 2x 4x -*
Ub-Clb2
Clb2
1 2 3 4 5 6
Unconjugated Clb2 remaining (percentage)
100
80
60
40
20
0
1 2 3 4 5 6
B
Acm1
APC - + + + + + +
Cdh1 + - + + + + +
Ubc4 + + + + + + +
Bmh1 - - - + + + +
Acm1 - - - - 1x 2x 4x
Ub-Clb2
Clb2
1 2 3 4 5 6 7
APC
Cdh1
Acm1 (1x)
Acm1 (2x)
Acm1 (4x)
α-FLAG
8 9 10 11 12
C
- GAL
+ GAL
1 2 3 4 5 6 7 8
Cdh1m9 + + + + + + + + -
Acm1 - + + + - + + + -
GAL - - - - + + + + -
1 2 3 4 5 6 7 8 9
α-FLAG
α-PGK
FLAG-Cdh1m9
FLAG-Acm1
PGK
D
Acm1
GST - + - - - -
GST-Clb2 - - + + + +
Cdh1 + + + + + -
Bmh1 - - - + + -
Acm1 - - - 2x 8x -
I E FT E FT E FT E E
α–FLAG
α–ProtA
FLAG-Cdh1
Acm1-TAP
1 2 3 4 5 6 7 8 9
Immobilized Protein
98
62
49
38
28
17
GST-Clb2
Control GST
E
WT
acm1Δ
Control
APC1-TAP
Control
APC1-TAP
APC1-TAP
I E FT E I E FT E E
α–FLAG
α–ProtA
FLAG-Cdh1
APC1-TAP
10 11 12 13 14 15 16 17 18

FIGURE 1.3

Figure 1.3: Acm1/Bmh1 inhibit APC^{Cdh1} ubiquitin ligase activity by blocking Cdh1 binding to the APC. A, Cdh1, Ubc4, and Clb2 used in this assay were produced in the TNT T7 Quick coupled *in vitro* transcription/translation kit and Clb2 was labeled with S^{35} methionine. APC was purified from yeast and Bmh1 from *E. coli.* Acm1 was purified from 2 L of asynchronous cells grown to OD_{600}=1 using 100 µl of washed calmodulin resin. Resin was washed extensively with APC-C buffer containing 1mM $CaCl_2$ and eluted in 2.5 mM EDTA buffer. 15 mM $MgCl_2$ was added to the eluted Acm1 before use in the ubiquitination assay. Components of the assay were mixed and incubated as described previously (36). Ubiquination of Clb2 can be seen as bands of decreasing gel mobility. The triangle represents increasing levels of Acm1. 1x Acm1 is the equivalent of 2 µl or 1/50 from the EDTA elution. Acm1 inhibits APC^{Cdh1} activity in a dose dependent manner. *Control purification from the *acm1Δ* strain equivalent to 4X concentration of Acm1 added. Levels of unconjugated Clb2 for the quantitation of APC^{Cdh1} inhibition by CAB complex formation are shown in the right panel. **B**, Ubiquitination assay is the same as above with the exception of using Cdh1 (p415ADH-FLAGCdh1 in YPL267WΔ) and Acm1 (pHLP107 in YGL003Δ) purified from yeast using FLAG immunoprecipitations for quantitation. Ubc4 and Clb2 produced in the *in vitro* transcription/translation kit. Bmh1 levels used in the assay are sufficient to see by Coomassie staining. Levels of APC, Cdh1, and Acm1 used in each assay can be seen in the lower panel. **C**, Cdh1 containing 9 of 11 CDK sites mutated to alanine was overexpressed through use of the *GAL* promoter (pNC219-FLAGCdh1m9 in YKA323). In the same strain, pHLP106 expressing FLAG-Acm1 from the *GAL* promoter was also transformed. Cells were plated on SD-Trp and grown for 48 hours. Colonies were selected and plated on Raf-Trp, grown for 48 hours and replica-plated to Raf-Trp media containing galactose. Extracts (40 µg) were separated by SDS-PAGE and immunoblotted with α-FLAG and PGK antibodies. Cdh1m9 overexpression is lethal when expressed without Acm1 (section 5 and lane 5), but lethality is rescued by Acm1 overexpression (sections 6 and 7, lanes 6 and 7). Three colonies were tested to ensure each contained both transformed plasmids. Sections 6, 7, and 8 contain both plasmids although section 8 exhibited low Cdh1m9 expression. Extract from control strain without plasmids present is shown in lane 9. **D,** Bacterially expressed GST-Clb2 was incubated with FLAG-Cdh1 from *acm1Δ* cells for 30 min. at 4° C after Cdh1 was pre-incubated with varying levels of Acm1. Clb2 was immobilized on affinity beads. GST bound to GSH beads and GST-Clb2 without Cdh1 were used as controls. Following incubation with Cdh1, columns were washed extensively with high salt APC-C buffer and eluted with 1X LDS sample buffer. Samples were run on a 4-12% Bis-Tris gel and transferred for Western blot analysis. Immobilized protein shown is Coomassie stained and is 20X greater than that shown in binding assay. I, FT, and E designate input, flow through, and elution. Antibodies used for detection are as noted. Clb2 binding to Cdh1 decreases with an increasing concentration of Acm1. **E,** N-terminally tagged FLAG-Cdh1 was purified from either WT or *acm1Δ* cells and incubated with APC via Apc1-TAP for 30 min. at 4° C. Empty calmodulin beads and APC-TAP without Cdh1 were used as controls. Following incubation with Cdh1, columns were washed extensively with high salt APC-C buffer and eluted with 1X LDS sample buffer. Samples were run on a 4-12% Bis-Tris gel and transferred for Western blot analysis. Immobilized protein shown is Coomassie stained and is 20X greater than that shown in binding assay. I, FT, and E designate input,

flow through, and elution. Antibodies used for detection are as noted. The presence of Acm1 has no effect on APC/Cdh1 binding.

A



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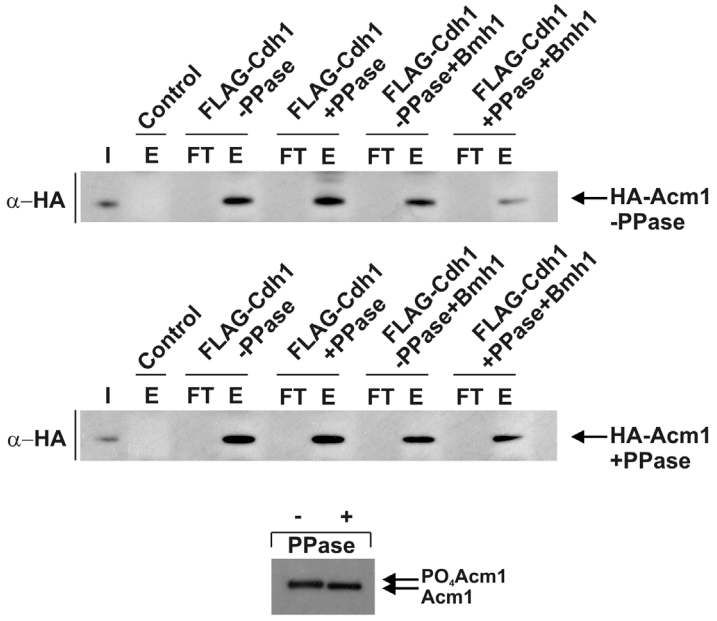


C

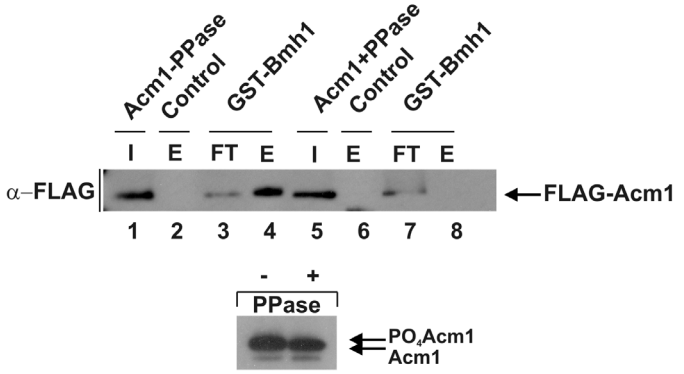
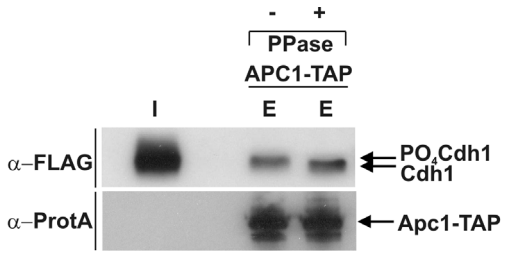


FIGURE 1.4

Figure 1.4: Phosphorylation is involved in the assembly of the CAB complex. **A,** An *in vitro* binding assay was used with C-terminally tagged Cdh1-FLAG purified from WT cells (YKA154) and treated with λ-phosphatase. Phosphatase treated and untreated Cdh1 was incubated with bacterially expressed GST-Bmh1 immobilized on GSH affinity beads for 2 hours at 4° C. Once Cdh1 was bound, columns were washed extensively with high salt APC-C buffer and eluted with 1X LDS sample buffer. I, FT, and E designate input, flow through, and elution. Phosphatase treatment of Cdh1 abolishes Bmh1 binding. **B,** An *in vitro* binding assay was used with N-terminally tagged FLAG-Cdh1 and HA-Acm1 treated with λ-phosphatase. FLAG-Cdh1 was purified from 2 L of asynchronous yeast cultures grown to OD_{600}=1 and incubated with 100 µl washed α-FLAG resin. Resin was divided and one half treated with phosphatase. Bound FLAG-Cdh1 was used as bait. HA-Acm1 was purified from 2 L of asynchronous cells grown to OD_{600}=1 and incubated with 50 µl of washed α-HA resin. One half was treated with phosphatase and eluted 2x in 50 µl low salt APC-C buffer with HA peptide at 100 µg/ml final concentration. 1/20 of HA-Acm1 elution was used in each assay and allowed to incubate for 1.5 hours at 4 C. Once bound, the proteins were washed extensively with high salt APC-C buffer. 40% of each reaction was loaded on the gel. A shift in dephosphorlation of Acm1 can be seen in the lower panel. Empty α-FLAG beads were used as the control. Phosphorylation of Acm1 or Cdh1 has no effect on their interaction with one another. **C,** An *in vitro* binding assay was used with N-terminally tagged and bacterially expressed GST-Bmh1. GST and GST-Bmh1 were prepared as described previously. N-terminally tagged FLAG-Acm1 was purified from 2 L of asynchronous yeast cultures grown to OD_{600}=1, purified using 50 µl α-FLAG resin, and one half treated with λ-phosphatase. FLAG-Acm1 was eluted with FLAG peptide (250 µg final conc.) in 50ul low salt APC-C buffer. Bound GST-Bmh1 (10 µl GSH beads) was used as bait and incubated for 30 min. at 4 C with either phosphatase treated or untreated FLAG-Acm1. 1/25 of FLAG-Acm1 elution was used in each assay. Bound proteins were washed extensively with high salt APC-C buffer and eluted in LDS sample buffer. 20 % from each assay was loaded on the gel. Nonphosphorylated Acm1 does not interact with Bmh1.

A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APC1-TAP
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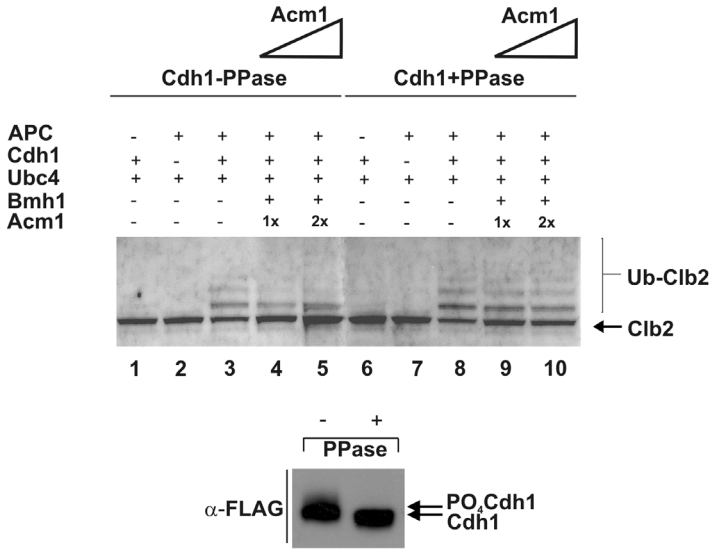
E E
α–FLAG
α–ProtA
PO4Cdh1
Cdh1
Apc1-TAP

B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Acm1
Cdh1-PPase
Cdh1+PPase
APC
Cdh1
Ubc4
Bmh1
Acm1
1x
2x
Ub-Clb2
Clb2
1 2 3 4 5 6 7 8 9 10
- +
PPase
α-FLAG
PO4Cdh1
Cdh1

FIGURE 1.5

Figure 1.5: Phosphorylated Cdh1 is inhibited by complex formation.
A, N-terminally tagged FLAG-Cdh1 was purified from *acm1Δ* cells and incubated with APC via Apc1-TAP immobilized on calmodulin beads. After incubation with Cdh1, the immobilized APC/bound Cdh1 was washed extensively, divided into two columns, and one treated with λ-phosphatase. After treatment, samples were separated on a 6% Tris-glycine gel to differentiate phosphorylated Cdh1 from unphosphorylated Cdh1 and analyzed by Western blot. Antibodies used for detection are as noted. Partially phosphorylated Cdh1 can bind the APC. **B,** Ubc4 and Clb2 used in this assay were produced by *in vitro* transcription/translation. N-terminally tagged FLAG-Cdh1 was purified from 2 L of asynchronous yeast cultures and incubated with 50 µl of α-FLAG resin. The beads were divided, washed extensively with APC-C buffer, and one half treated with λ-phosphatase for dephosphorylation. 1/50 of the FLAG-Cdh1 elution was used in each assay. Acm1 was purified from 2 L of asynchronous cells grown to OD_{600}=1 using 100 µl of washed calmodulin resin. Resin was washed extensively with APC-C buffer containing 1mM $CaCl_2$ and eluted in 2.5 mM EDTA buffer. 15 mM $MgCl_2$ was added to the eluted Acm1 before use in the ubiquitination assay. The triangle represents increasing levels of Acm1. 1x Acm1 is the equivalent of 2 µl or 1/50 from the EDTA elution. Each reaction contains components designated by +/-. Components of the assay were mixed and incubated as described previously. Ubiquination of Clb2 can be seen as bands of decreasing gel mobility. Partially phosphorylated Cdh1 activates the APC, and Acm1 and Bmh1 inhibit phosphorylated Cdh1 preferentially over nonphosphorylated Cdh1.

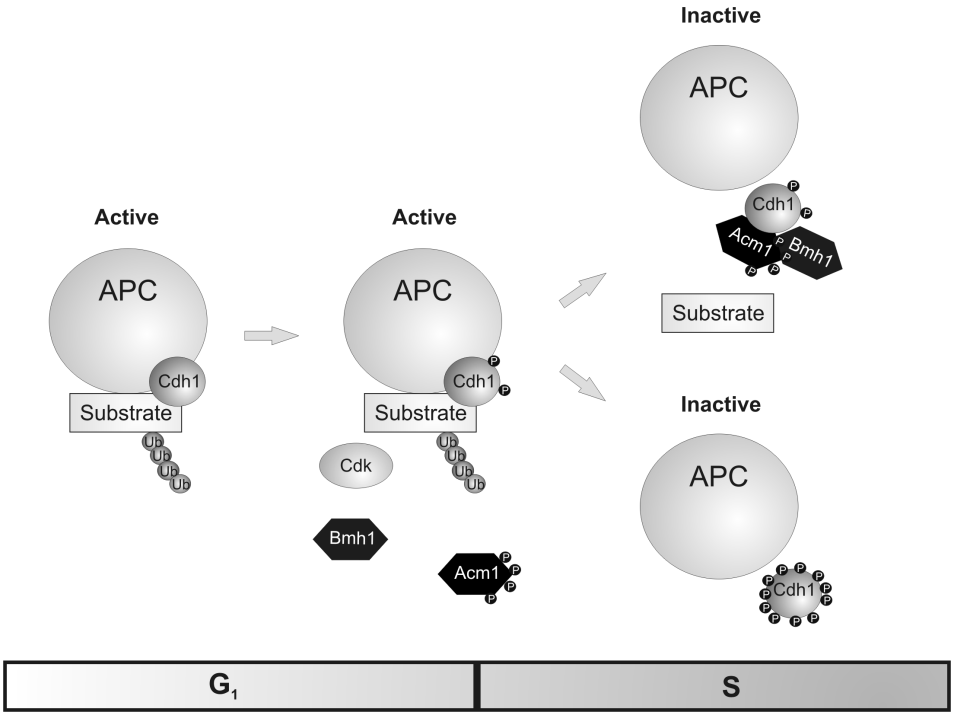


FIGURE 1.6

Figure 1.6: Model of APC^{Cdh1} inactivation.
APC^{Cdh1} is active throughout G1 phase until Cdh1 is phosphorylated by the cyclin dependent kinase Cdc28 at the G1/S phase transition. Additionally, a complex forms with the co-activator consisting of Acm1 and Bmh1, which inhibits the APC at the end of G_1. The CAB complex dissociates the substrate from Cdh1 and acts independent of Cdh1 phosphorylation for the timely inactivation of the APC.

CHAPTER 3

Mechanism for APC^{Cdh1} inhibition by the substrate-like inhibitor Acm1

Overview

The anaphase-promoting complex (APC) catalyzes the polyubiquitination of numerous proteins that targets them for degradation by the proteasome at specific times during the cell cycle. The Cdh1 and Cdc20 proteins are coactivators that are also required for APC activity. In the previous chapter, we reported the identification of Acm1 as an inhibitor of the budding yeast APC both *in vitro* and *in vivo* that associates with Cdh1 and the 14-3-3 homologs Bmh1 and Bmh2 in a stable, cell cycle-regulated complex. Here we further characterize the Acm1 protein and present a model for how Acm1 inhibits APC. Recombinant Acm1 alone was qualitatively as effective an inhibitor of Clb2 ubiquitination *in vitro* as native Acm1 purified from yeast in the presence of the 14-3-3 proteins. Thus, although Acm1 is heavily phosphorylated *in vivo*, neither phosphorylation nor 14-3-3 binding is required for its function as an APC inhibitor. Acm1 was specific for the Cdh1-dependent form of APC (APC^{Cdh1}) because it potently inhibited APC^{Cdh1}-catalyzed ubiquitination of Pds1 but had no effect on APC^{Cdc20}-catalyzed Pds1 ubiquitination, even at much higher concentrations. Acm1 contains conserved D-box and KEN sequences that are common to many APC^{Cdh1} substrates and it is also rapidly degraded in late M phase like many APC targets. We found that mutations in the D-box and KEN sequences prevent association of Acm1 with Cdh1 in a co-immunoprecipitation assay, and strongly suppress its ability to inhibit APC both *in vitro* and *in vivo*. However, we observed no evidence that Acm1 itself was ubiquitinated during reactions in which it completely inhibited Clb2 ubiquitination, and D-box/KEN mutants were not stabilized at all *in vivo*. We have discovered regions of the N-terminus

and C-terminus of Acm1 that prohibit it from being processed as a substrate. These data suggest that the interaction between Acm1 and Cdh1 may be structurally similar to Cdh1-subtrate interactions but that full length Acm1 is not processed as a substrate. We therefore propose that Acm1 is a substrate mimic that inhibits APC by competitive exclusion of substrate binding to Cdh1.

Introduction

Control of the eukaryotic cell cycle involves a network of tightly regulated machinery that governs cell cycle progress. Progression through the cell cycle is largely controlled by cyclin dependent kinases (CDKs) and the timely degradation of proteins. The periodic accumulation and destruction of cyclins at key points during the cell cycle activate or inactivate CDKs respectively. Ubiquitin-mediated proteolysis of cyclins is one way eukaryotic cells keep proper cyclin levels in check (1-3). The anaphase-promoting complex (APC) is an E3 ubiquitin ligase whose responsibility is to mediate the degradation of cyclins and other regulatory proteins during the cell cycle. The APC consists of 13 subunits in yeast with the catalytic core consisting of a cullin homolog APC2 and a RING-H2 finger protein APC11 (108).

Although the APC is present constitutively throughout the cell cycle, APC activity is confined to early M phase through G_1. APC activity is triggered by the presence of one of two WD40 repeat proteins in mitosis known as Cdc20 and Cdh1 by forming distinct complexes, APC^{Cdc20} or APC^{Cdh1}. Cdc20 and Cdh1 control substrate specificity by associating with particular substrates at certain times throughout the cell

cycle (25,29,35). Cdc20 activates the APC during M phase and triggers the metaphase to anaphase transition after marking Pds1 for degradation (13). Cdh1 activates the APC once it is dephosphorylated by Cdc14 phosphatase at the end of M phase (87). APC^{Cdh1} activity leads to the onset and maintenance of G_1 and prevents premature S phase entry (34).

Regulation of APC^{Cdc20} involves targeting Cdc20 for degradation by APC^{Cdh1} (60). Means of APC^{Cdh1} regulation include the phosphorylation of Cdh1 by Clb5-CDK at the end of G_1 rendering APC^{Cdh1} inactive (57). Other means of Cdh1 regulation involve its re-localization to the cytoplasm presumably to remove Cdh1 from the vicinity of the APC (109). In vertebrates, Emi1 has been shown to bind and inhibit Cdh1 by mimicking an APC substrate (63). Other protein-protein interactions have been described in the regulation of both APC^{Cdc20} and APC^{Cdh1} although until recently, no known inhibitors for APC^{Cdh1} had been discovered in yeast (69,71,110).

In the previous chapter, we reported a new protein-protein interaction in yeast between Cdh1 and Acm1 (111,112). The function of Acm1 was characterized as an APC^{Cdh1} inhibitor independent of Cdh1 phosphorylation. Acm1 forms a complex with Cdh1 and members of the 14-3-3 family of proteins, Bmh1 and Bmh2, in late G_1 throughout S phase. Acm1 levels oscillate during the cell cycle and are highest in S phase. The presence of phosphorylated Acm1 is required for the complex to form, thus complex assembly is restricted to the cell cycle window in which Acm1 is expressed. Upon the addition of Acm1 and Bmh1, APC^{Cdh1} activity decreases *in vitro* in a dose dependent manner. Additionally, we showed overexpression of Acm1 could rescue the

lethality caused by overexpressing WT Cdh1 or the constitutively active Cdh1 phosphomutant.

Acm1 functions as an inhibitor by blocking Clb2 binding to Cdh1 but not binding to the APC. Although Acm1 has been shown to be an effective APC^{Cdh1} inhibitor, the mechanism and specificity of inhibition are not clear. Here, we report additional properties of APC^{Cdh1} inhibition by Acm1, providing a clearer picture of the primary mechanism of action of Acm1. We demonstrate that neither the presence of the 14-3-3 proteins nor Acm1 phosphorylation is necessary for inhibition of APC^{Cdh1} activity by Acm1. Acm1 contains APC substrate-like regions known as D boxes and a KEN box that when mutated abolish the interaction with Cdh1. The C-terminus of Cdh1, containing the WD40 repeat domains implicated in substrate binding, is also the region of Acm1 binding (38). Although Acm1 binds the C-terminal region of Cdh1 like other APC substrates, it is not ubiquitinated by the APC until the N-terminus and C-terminus are truncated. Together, we suggest Acm1 functions as an APC^{Cdh1} inhibitor by mimicking an APC substrate.

Experimental Procedures

Plasmid construction. Mutations in the D-box and KEN sequences of *ACM1* were created in pHLP117 (112) using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). All D-box mutations resulted in RxxL to AxxA substitutions, and the KEN box was changed to AAA. The five CDK consensus phosphorylation sequences in Acm1 were changed to alanine in similar fashion and subcloned with a single HA epitope into

the XbaI and XhoI sites of p415GAL1 to create pHLP110 expressing an N-terminal HA epitope fusion from the galactose-inducible *GAL1* promoter. *ACM1* with a 3XFLAG epitope was cloned into the *NcoI* and *NotI* sites in the pIVEX-2.3d vector (Roche) for bacterial expression.

APC Purification. 2 L of cells from YKA156 were grown to OD_{600}=0.5 at 25° C and temperature shifted to 37° C for 2 hours. Cells were lysed in 1 volume of APC-C lysis buffer (25mM HEPES-NaOH pH 7.5, 400mM NaCl, 10% glycerol, 0.1% Triton X-100, 0.1mM dithothreitol, 0.5mM PMSF, and complete protease inhibitor cocktail (Roche)) by vortexing for 45 min. at 4° C in 1.7 ml microcentrifuge tubes containing 0.5 ml 0.5 mm glass beads. Cell lysate was cleared by centrifugation at 16,000 x g for 15 min. Soluble extracts were pooled and cleared a second time at 5000 rpm for 5 min. Extracts from YKA156 were incubated with EZView anti-FLAG M2 antibody-coupled resin (Sigma) for 1.5 hrs. at 4° C, washed extensively with APC-C buffer and eluted 2X with FLAG peptide (250 µg/ml) in low salt APC-C buffer (25mM HEPES-NaOH pH 7.5, 150mM NaCl, 10% glycerol, 0.1% Triton X-100, 0.1mM dithothreitol).

Acm1 purification. Overnight cultures of *E. coli* harboring *ACM1* in the pIVEX-2.3d vector were grown and used to inoculate 1L of 2XYT (16g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl) containing 100 µg/ml ampicillin. Bacterially expressed 3XFLAG-Acm1 was grown at 37° C to OD_{600}=0.8 and IPTG added to induce protein expression. Cells were grown for three additional hours and harvested. Cell pellet was washed with water and resuspended in 4 volumes of cold lysis buffer (50mM $NaPO_4$ pH 7.5, 500mM

NaCl, 10% glycerol) on ice. Lysozyme was added to 1 mg/ml and incubated on ice for 30 min. Triton X-100 was added to 1% along with PMSF to 1mM and pepstatin to 1 µM. Mixture was sonicated with brief pulses until the viscosity was reduced to water-like consistency. Lysate was spun at 35,000 x g for 30 min. Supernatant was incubated with 500 µl of anti-FLAG resin and incubated for 1 hour at 4° C. Resin was washed 4X with 25 ml lysis buffer. Acm1 was eluted 2X with 250 µg/ml of 3XFLAG peptide. Acm1 purified from yeast using pHLP117 and pHLP149 (3D box/KEN mutant) transformed into *acm1Δ* cells (YKA150 background) were grown asynchronously to OD_{600}=1 and lysed in 1 volume APC-C lysis buffer in the same manner as for APC. Cell extracts were incubated with 100 µl of EZView anti-HA antibody-coupled resin (Sigma) for 1.5 hrs at 4° C. Resin was 2X with APC-C buffer and then 2X with low salt APC-C buffer. Bound protein was eluted with 100 µg/ml HA peptide in low salt APC-C buffer. Acm1 (pRSET-FLAG-Acm1) used in the ubiquitination assay was made using the TNT T7 Quick coupled *in vitro* transcription/translation kit (Promega).

Co-IP and Western blotting. Coimmunopurification (Co-IP) experiments testing interactions between 3FLAG-Cdh1 and D-box/KEN mutant HA-Acm1 were performed as described by M. Hall (112). Briefly, soluble yeast extracts were incubated with anti-FLAG M2 resin (Sigma) to bind 3FLAG-Cdh1. Resin was washed with Buffer C (50mM sodium phosphate [pH 7.5], 100mM NaCl, 10% glycerol, 0.1% Triton X-100, 50mM β-glycerophosphate) supplemented with 0.5mM dithiothreitol, 0.5mM phenylmethylsulfonyl fluoride, 1µM pepstatin, 100µM leupeptin, and 5mM EDTA, and 3FLAG-Cdh1 eluted with 250 µg/ml 3FLAG peptide (Sigma). FLAG and HA signals in

the eluate were measured by immunblotting.

Cell-cycle expression profiles of D-box/KEN mutant Acm1. W1588-4c containing plasmids with wild-type or D-box/KEN mutant 3HA-Acm1 expressed from the natural *ACM1* promoter were synchronized with α-factor or nocodazole, released from arrest and the expression level of Acm1 monitored at incremental timepoints by immunoblotting as described by M. Hall (112).

Ubiquitination assay. Ubiquitination assays were carried out as described by D. Barford (36). The substrates Clb2 (pRSETClb2) and Pds1 (pRSETPds1), the co-activators Cdh1 (pET28-His_6-Cdh1) and Cdc20 (pET28-His_6-Cdc20), and the E2 Ubc4 (pET28-His_6-Ubc4) were prepared using the TNT T7 Quick coupled *in vitro* transcription/translation kit (Promega). APC (YKA156) and Acm1 were purified from budding yeast (see APC and Acm1 purification). APC used in each reaction was 1/50 of the elution from 2 L of cells. Clb2, Pds1, and Acm1 were radiolabeled with S^{35} methionine (Perkin Elmer). Reactions were run on a 4-12% Bis-Tris NuPAGE gel (Invitrogen), dried, and imaged using Hyperfilm (Amersham Biosciences).

***In vivo* APC/C inhibition assay.** Inhibition of APC/C^{Cdh1} activity *in vivo* was determined by monitoring the effect of overexpression of *ACM1* alleles on the toxicity of Cdh1 overexpression as described by M. Hall (112).

Results

Acm1 does not require the 14-3-3 proteins or phosphorylation to act as a specific APC^{Cdh1} inhibitor. Previously, we identified Acm1 in a Cdh1 pulldown in a complex with Bmh1 and Bmh2. The interactions of Bmh1 and Bmh2 were dependent on the presence of phosphorylated Acm1 while the formation of the Cdh1/Acm1/Bmh1 complex was shown to inhibit APC^{Cdh1} activity. Additionally, we observed no inhibition of APC activity with the addition of Bmh1 alone. Since Acm1 mediates the interaction between the 14-3-3 proteins and Cdh1, the interaction between Acm1 and Cdh1 was examined in greater detail. To decipher whether Acm1 could act as an inhibitor without the 14-3-3 proteins or phosphorylation, Acm1 was purified from *E. coli* and tested for its ability to inhibit APC^{Cdh1} activity in an *in vitro* ubiquitination assay. Non-phosphorylated Acm1 from *E. coli* in the absence of 14-3-3 proteins inhibited APC^{Cdh1} activity *in vitro* in a dose-dependent manner using Clb2 as a substrate (Figure 2.1A). Levels of Acm1 used were comparable to those used in the previous chapter for inhibition of APC^{Cdh1} (Figure 1.3B). In addition, we made an Acm1 mutant with all 5 consensus CDK sites mutated to alanine to see if overexpression of this mutant could rescue the lethality caused by Cdh1 overexpression *in vivo*. As seen in Figure 2.1B, overexpression of Cdh1 alone is lethal while both WT and the Acm1 CDK mutant can rescue this lethality. The Acm1 CDK mutant does not interact with Bmh1 and Bmh2 in co-immunoprecipitation experiments further emphasizing that the 14-3-3 proteins are not needed for APC inhibition (data not shown). Together, our results demonstrate that the primary mechanism for APC^{Cdh1} inhibition is through Acm1 binding to Cdh1 and that neither Bmh1 or Bmh2 nor Acm1 phosphorylation is required for this inhibition.

The Acm1 KEN box and D box regions mediate the interaction with Cdh1. Both KEN boxes and D boxes have been shown to be involved in substrate interactions with Cdh1 and Cdc20 (33,37). Interestingly, Acm1 contains three D boxes and one KEN box in *Saccharomyces cerevisiae* with the first and third D box regions and the KEN box being highly conserved in other budding yeast species (Figure 2.2A). Since Acm1 binds directly to Cdh1, we questioned which regions of Acm1 were involved in the interaction with Cdh1. To test this, we mutated each of the three consensus D box regions and the KEN box region to systematically decipher if any of these regions singly or in combination affected the Acm1/Cdh1 interaction. Immunoprecipitations of Cdh1 were performed from cells co-expressing each of the different Acm1 mutants. Mutations in the third D box and the KEN box decreased the binding affinity of Acm1 to Cdh1 (Figure 2.2B, lanes 3 and 4). With both the third D box and KEN box mutated together, binding between Acm1 and Cdh1 was completely abolished (Figure 2.2B lane 7). *In vivo*, the double mutant (3D box and KEN box) was only able to partially rescue lethality caused by Cdh1 overexpression (Figure 2.2C). The double mutant was also used in the *in vitro* ubiquitination assay to test whether the mutant could still act as an APC^{Cdh1} inhibitor. In comparison to WT Acm1, there was no decrease in Clb2 ubiquitination by the mutant Acm1 (Figure 2.2D). Therefore, the third D box and KEN box of Acm1 are required for its interaction with Cdh1 and its ability to act as an inhibitor.

Acm1 binds the WD40 repeat containing region of Cdh1. The WD40 propeller domain of Cdh1 has been shown to be the D box receptor for APC substrates (38). Since

Acm1 contains substrate-like D boxes and a KEN box, we hypothesized Acm1 should bind to the WD40 domain of Cdh1. In order to test this, we generated an N-terminal fragment (aa 1-249) of Cdh1 containing the prominent CDK phosphorylation sites and a C-terminal fragment (aa 241-end) containing the WD40 domains. Immumoprecipitations of with each Cdh1 truncation resulted in Acm1 binding to only the Cdh1 C-terminal fragment (Figure 2.3A). Additionally, the C-terminal fragment of Cdh1 is capable of pulling down Bmh1 and Bmh2 in addition to Acm1 (Figure 2.3B).

In Chapter 2, we discussed the decrease in binding we observed between Cdh1 and Clb2 upon the addition of increasing Acm1 concentrations. This result suggested Acm1 out-competed Clb2 for Cdh1 binding. Together, with the substrate like regions of Acm1 and the Cdh1 substrate binding region involved in this interaction, our results suggest Acm1 acts as a competitive, substrate-like inhibitor.

Acm1 is a specific inhibitor of the Cdh1-dependent APC and is not ubiquitinated by the APC. Acm1 was first identified as a Cdh1 interacting protein and APC^{Cdh1} inhibitor. Because Acm1 could inhibit APC^{Cdh1} activity, our next question was whether Acm1 could also inhibit APC^{Cdc20} as a multifunctional APC inhibitor. To test this notion, Acm1 levels 5X greater than those used to efficiently inhibit APC^{Cdh1} activity were added to an *in vitro* APC^{Cdc20} ubiquitination assay. Acm1 did not inhibit Pds1 ubiquitination by APC^{Cdc20} but clearly inhibited APC^{Cdh1} mediated Pds1 ubiquitination (Figure 2.4A, lanes 3 and 6).

Since Acm1 appears to act as a specific Cdh1 inhibitor by competing for substrate binding to the co-activator, we questioned whether Acm1 could be ubiquitinated or

degraded in an APC specific manner while acting as an inhibitor. In order to test this hypothesis, we employed an *in vitro* ubiquitination assay using the APC^{Cdh1} complex. APC^{Cdh1} does not appear to be able to ubiquitinate full length Acm1 *in vitro* although Acm1 levels are sufficient for inhibition (Figure 2.4B, lanes 3 and 5). Truncations of Acm1 were made to test whether certain regions prohibited Acm1 from being processed as an APC substrate. When both the N-terminus and C-terminus of Acm1 are truncated, Acm1 is ubiquitinated by APC^{Cdh1} suggesting these regions are responsible for the ability of Acm1 to serve as an APC inhibitor (Figure 2.4C, 42-140). Additionally, the 3D box/KEN box mutant of Acm1, previously shown to completely disrupt binding to Cdh1, was transformed into *acm1Δ* cells and a block/release time course performed to measure the stability of the Acm1 mutants compared to WT Acm1. No difference was observed in Acm1 stability between the Acm1 mutants and WT Acm1 in a G_1 phase arrest and release experiment further suggesting APC does not play a role in Acm1 degradation (Figure 2.4D). These results also suggest that Acm1 mimicks an APC substrate in order to serve as a potent APC^{Cdh1} inhibitor.

Discussion

Upon identifying Acm1 in Cdh1 immunoprecipitations, our immediate assumption was that Acm1 was a novel APC^{Cdh1} substrate. Further investigation revealed Acm1 functioned to inhibit APC^{Cdh1} activity by blocking substrate binding to Cdh1. Acm1 does contain APC substrate-like D boxes and a KEN box, and by mutating these substrate-like regions, the interaction between Acm1 and Cdh1 was abolished. In

addition, we found Acm1 binds to the substrate binding WD40 repeat region of Cdh1. Therefore, Acm1 looks like an APC substrate but is an APC inhibitor. The question is then raised, could Acm1 be both a novel APC substrate and APC inhibitor simultaneously? Acm1 levels are highest during S phase and lowest during M phase and G_1. If Acm1 was degraded during M phase by ubiquitin-mediated proteolysis, APC^{Cdc20} could be the likely E3 ligase responsible. In order to investigate the possibility of Acm1 acting as both an inhibitor and substrate, we employed an *in vitro* ubiquitination assay using the radiolabled substrates Pds1 and Clb2 and radiolabled Acm1. As a result Acm1 does not act as an inhibitor of APC^{Cdc20} suggesting Acm1 does not block substrate binding to Cdc20 as it does with Cdh1. Although Acm1 acts as an inhibitor of APC^{Cdh1}, it does not appear to be ubiquitinated as an APC^{Cdh1} substrate *in vitro* until both the N-terminus and C-terminus are removed. When these regions were truncated, Acm1 is converted to an APC^{Cdh1} substrate. Additionally, the pattern of Acm1 oscillation during cell cycle progression did not change when using the Acm1 3D box/KEN box double mutant. Because D box and KEN box regions are present in both APC^{Cdc20} and APC^{Cdh1} substrates, this data further suggests Acm1 is not likely an APC substrate. Acm1 appears to function solely as an APC^{Cdh1} inhibitor and may be targeted for degradation by other means.

We have shown the ability of Acm1 to inhibit APC^{Cdh1} activity is not enhanced by the presence of the 14-3-3 proteins, Bmh1 and Bmh2 or by Acm1 phosphorylation. An interesting question is to why the 14-3-3 proteins are present in a complex with Cdh1 and Acm1. Although the roles for 14-3-3 proteins range from signal transduction to the relocalization of proteins, they have been commonly shown to bind phosphoproteins

(93,113). We have shown phosphorylation of Acm1 is required for the 14-3-3 proteins to bind. Because we have also observed instability of Acm1 with all CDK sites mutated to alanine in cells (data not shown) and a down shift in gel mobility prior to its disappearance in the cell cycle (Chapter 2, Figure 1.2C), we believe one possible role for the 14-3-3 proteins may be to protect Acm1 from premature degradation. However, further examination is required to elucidate the role of Bmh1 and Bmh2.

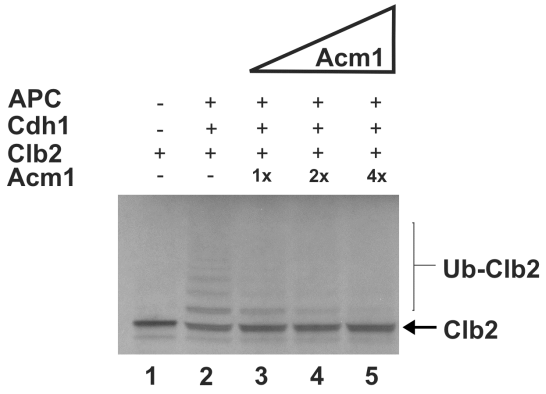
Acm1 appears to act as an APC^{Cdh1} inhibitor by mimicking an APC substrate. Evolutionarily, this mechanism makes complete sense to out-compete substrate binding in order to shutoff enzymatic activity. However, proteins mimicking substrates as a means of inhibition is not a novel concept and has been illustrated in several different systems. The phosphoprotein, hnRNP-U, has been shown to occupy the receptor subunit of the $SCF^{\beta\text{-}TrCP}$ as a pseudosubstrate perhaps as a way to block substrate binding to the SCF (114). Additionally, the BPS region of Grb14, a tissue-specific negative regulator of insulin receptor signaling, binds as a psuedosubstrate inhibitor (115). In vertebrates, Emi1 has been characterized as mimicking an APC substrate to inhibit APC^{Cdh1} (63). Although Acm1 and Emi1 act as APC^{Cdh1} inhibitors by mimicking APC substrates, there are key differences between the two proteins. When Emi1 is phosphorylated, it is converted to a SCF substrate. Although we have not yet determined how Acm1 levels are regulated during the cell cycle, we do know Acm1 phosphorylation aids in its stability. In addition, Acm1 and Emi1 share no sequence homology. Evolutionarily, it is possible as these species diverged, the same need was present to inhibit APC^{Cdh1} by additional means other than phosphorylation. Potentially, what once was an APC substrate was converted to an inhibitor through mutation of one or more regions of the

substrate. However, further experiments are needed to determine the ultimate fate of Acm1.

Table 2
S. cerevisiae strains used in Chapter 3

Strain	Relevant Genotype	Source
W1588-4c	*MAT*a *ade2-1 can1-100 His3-11 15 leu2-3 112 trp1-1 ura3-1*	R. Rothstein
YKA150	W1588-4c *bar1Δ::URA3*	Hall et al. 2004
YKA254	W1588-4c *acm1Δ*::KanMX4	This Study
YKA323	YKA150 *acm1Δ*::KanMX4	This Study
BY4741	*MAT*a *his3-D1 leu2-D0 met15-D0 ura3-D0*	Open Biosystems
YKA245	BY4741 *cdh1Δ*::KanMX4 3HA-Acm1 *bar1Δ::URA3*	This Study
YKA237	BY4741 *acm1Δ*::KanMX4	This Study
YKA294	BY4741 *bar1Δ::HisG* 3FLAG-Cdh1 *acm1Δ*::KanMX4	This Study

A


Acm1
APC - + + + +
Cdh1 - + + + +
Clb2 + + + + +
Acm1 - - 1x 2x 4x
Ub-Clb2
Clb2
1 2 3 4 5

B

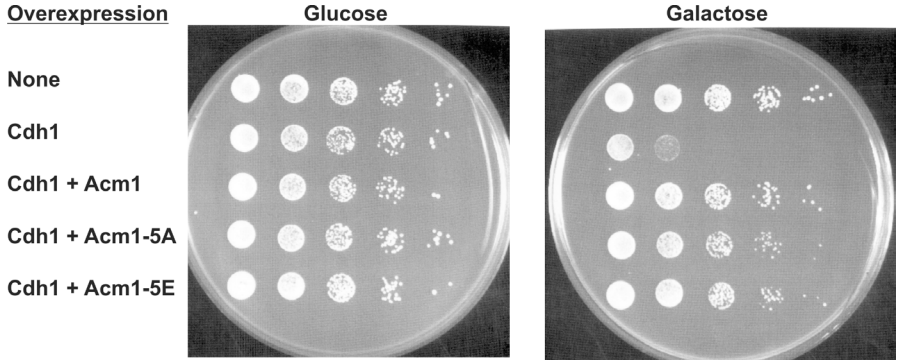

Overexpression
Glucose
Galactose
None
Cdh1
Cdh1 + Acm1
Cdh1 + Acm1-5A
Cdh1 + Acm1-5E

FIGURE 2.1

Figure 2.1: Acm1 does not require 14-3-3 proteins or phosphorylation to act as an APC inhibitor.
A, Cdh1, Ubc4, and Clb2 used in this assay were produced in the TNT Quick coupled *in vitro* transcription/translation kit. Clb2 was labeled with S^{35} methionine. APC was purified from yeast extracts while Acm1 was purified from *E. coli.* Ubiquitinated Clb2 can be seen as bands of decreasing gel mobility. Nonphosphorylated Acm1 inhibits APC^{Cdh1} activity in a dose-dependent manner. **B,** Liquid YKA150 cultures containing empty control plasmids or single-copy GAL1 expression plasmids (pHLP163 for *3FLAG-CDH1*, pHLP109 for *HA-ACM1*, pHLP110 for *HA-ACM1*-5A, and pHLP111 for *HA-ACM1*-5E) were grown in selective raffinose medium, and 10-fold serial dilutions were spotted and grown on selective medium containing either glucose or galactose. Acm1 5A has all five consensus CDK sites on Acm1 mutated to alanine. Acm1 5E has all five consensus CDK sites mutated to glutamic acid. Both Acm1 CDK mutants inhibit APC^{Cdh1} activity *in vivo*.

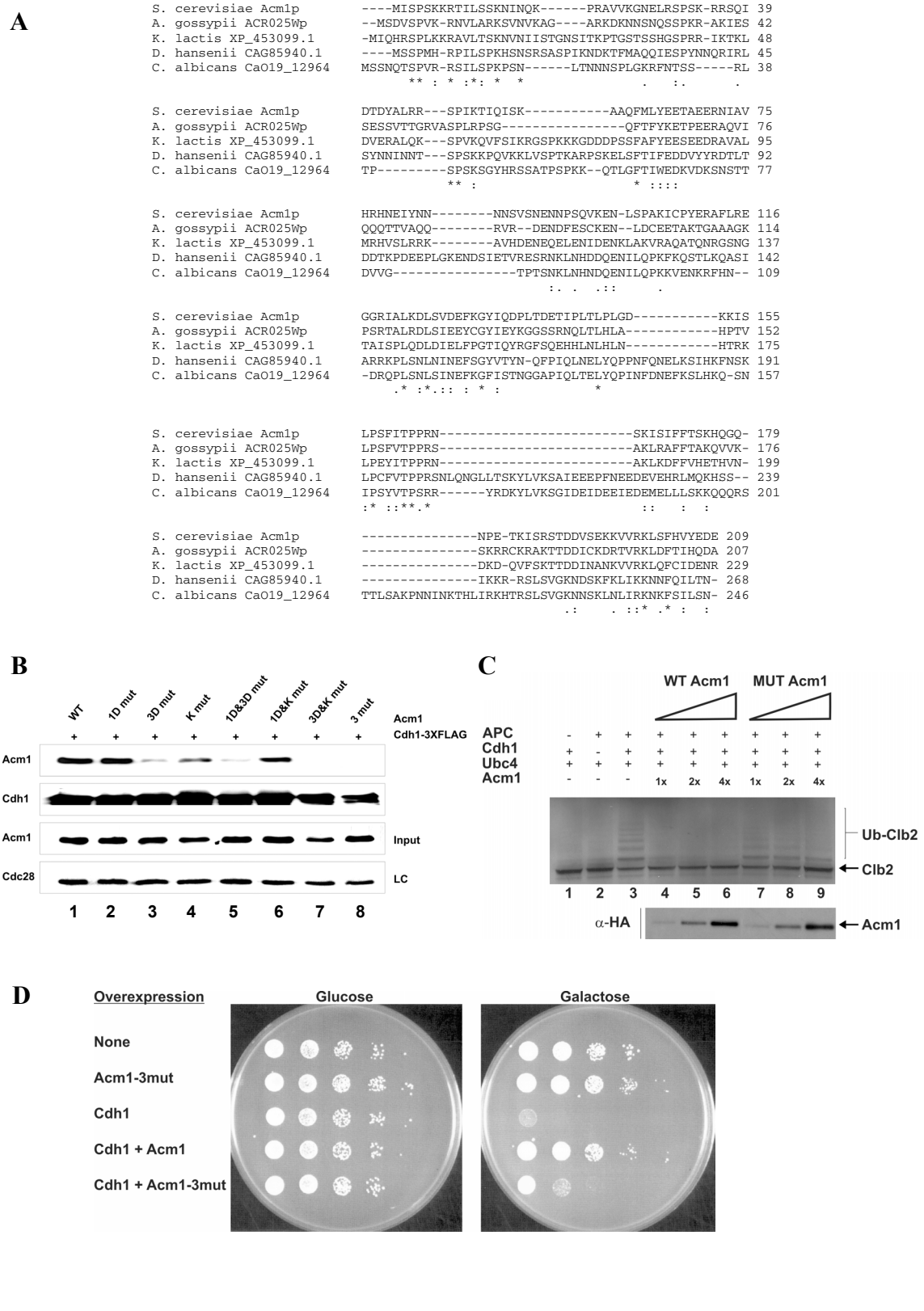

A
S. cerevisiae Acm1p ----MISPSKKRTILSSKNINQK------PRAVVKGNELRSPSK-RRSQI 39
A. gossypii ACR025Wp --MSDVSPVK-RNVLARKSVNVKAG----ARKDKNNSNQSSPKR-AKIES 42
K. lactis XP_453099.1 -MIQHRSPLKKRAVLTSKNVNIISTGNSITKPTGSTSSHGSPRR-IKTKL 48
D. hansenii CAG85940.1 ----MSSPMH-RPILSPKHSNSRSASPIKNDKTFMAQQIESPYNNQRIRL 45
C. albicans CaO19_12964 MSSNQTSPVR-RSILSPKPSN------LTNNNSPLGKRFNTSS-----RL 38
S. cerevisiae Acm1p DTDYALRR---SPIKTIQISK----------AAQFMLYEETAEERNIAV 75
A. gossypii ACR025Wp SESSVTTGRVASPLRPSG---------------QFTFYKETPEERAQVI 76
K. lactis XP_453099.1 DVERALQK---SPVKQVFSIKRGSPKKKGDDDPSSFAFYEESEEDRAVAL 95
D. hansenii CAG85940.1 SYNNINNT---SPSKKPQVKKLVSPTKARPSKELSFTIFEDDVYYRDTLT 92
C. albicans CaO19_12964 TP---------SPSKSGYHRSSATPSPKK--QTLGFTIWEDKVDKSNSTT 77
S. cerevisiae Acm1p HRHNEIYNN--------NNSVSNENNPSQVKEN-LSPAKICPYERAFLRE 116
A. gossypii ACR025Wp QQQTTVAQQ--------RVR--DENDFESCKEN--LDCEETAKTGAAAGK 114
K. lactis XP_453099.1 MRHVSLRRK--------AVHDENEQELENIDENKLAKVRAQATQNRGSNG 137
D. hansenii CAG85940.1 DDTKPDEEPLGKENDSIETVRESRNKLNHDDQENILQPKFKQSTLKQASI 142
C. albicans CaO19_12964 DVVG----------------TPTSNKLNHNDQENILQPKKVENKRFHN-- 109
S. cerevisiae Acm1p GGRIALKDLSVDEFKGYIQDPLTDETIPLTLPLGD----------KKIS 155
A. gossypii ACR025Wp PSRTALRDLSIEEYCGYIEYKGGSSRNQLTLHLA----------HPTV 152
K. lactis XP_453099.1 TAISPLQDLDIELFPGTIQYRGFSQEHHLNLHLN----------HTRK 175
D. hansenii CAG85940.1 ARRKPLSNLNINEFSGYVTYN-QFPIQLNELYQPPNFQNELKSIHKFNSK 191
C. albicans CaO19_12964 -DRQPLSNLSINEFKGFISTNGGAPIQLTELYQPINFDNEFKSLHKQ-SN 157
S. cerevisiae Acm1p LPSFITPPRN-------------------------SKISIFFTSKHQGQ- 179
A. gossypii ACR025Wp LPSFVTPPRS-------------------------AKLRAFFTAKQVVK- 176
K. lactis XP_453099.1 LPEYITPPRN-------------------------AKLKDFFVHETHVN- 199
D. hansenii CAG85940.1 LPCFVTPPRSNLQNGLLTSKYLVKSAIEEEPFNEEDEVEHRLMQKHSS-- 239
C. albicans CaO19_12964 IPSYVTPSRR------YRDKYLVKSGIDEIDEEIEDEMELLLSKKQQQRS 201
S. cerevisiae Acm1p ---------------NPE-TKISRSTDDVSEKKVVRKLSFHVYEDE 209
A. gossypii ACR025Wp ---------------SKRRCKRAKTTDDICKDRTVRKLDFTIHQDA 207
K. lactis XP_453099.1 ---------------DKD-QVFSKTTDDINANKVVRKLQFCIDENR 229
D. hansenii CAG85940.1 ---------------IKKR-RSLSVGKNDSKFKLIKKNNFQILTN- 268
C. albicans CaO19_12964 TTLSAKPNNINKTHLIRKHTRSLSVGKNNSKLNLIRKNKFSILSN- 246
B
WT
1D mut
3D mut
K mut
1D&3D mut
1D&K mut
3D&K mut
3 mut
Acm1
Cdh1-3XFLAG
Acm1
Cdh1
Acm1
Cdc28
Input
LC
1 2 3 4 5 6 7 8
C
WT Acm1
MUT Acm1
APC
Cdh1
Ubc4
Acm1
1x 2x 4x 1x 2x 4x
Ub-Clb2
Clb2
1 2 3 4 5 6 7 8 9
α-HA
Acm1
D
Overexpression
Glucose
Galactose
None
Acm1-3mut
Cdh1
Cdh1 + Acm1
Cdh1 + Acm1-3mut

FIGURE 2.2

Figure 2.2: The Acm1 KEN box and 3-D box regions mediate the interaction with Cdh1.

A, ClustalW sequence alignment of D-box and KEN box regions (highlighted in yellow) and consensus CDK sites (S/T-P-X-K/R) highlighted in grey with other yeast species. **B,** Co-immunoprecipitation with Cdh1 and Acm1 D/KEN box mutants. 1D mutant contains the mutation in the 1st D box of Acm1. 3D contains mutations in the 3rd D box of Acm1. K mut contains a mutation in the KEN box of Acm1. 3mut contains mutations in the 1st and 3rd D box regions as well as in the KEN box of Acm1. The 3D and KEN box mutations together abolish interaction with Cdh1. **C,** *In vitro* ubiquitination assay using 3D/KEN box Acm1 mutant verses WT Acm1. Cdh1, Ubc4, and Clb2 used in this assay were produced in the TNT Quick coupled *in vitro* transcription/translation kit. Clb2 was labeled with S^{35} methionine. APC and Acm1 were purified from yeast extracts as described. Ubiquitinated Clb2 can be seen as bands of decreasing gel mobility. WT Acm1 inhibits APC^{Cdh1} activity but the Acm1 mutant does not. **D,** Liquid YKA150 cultures containing empty control plasmids or single-copy GAL1 expression plasmids pHLP163 for *3FLAG-CDH1*, pHLP109 for *HA-ACM1*, and pHLP126 for *HA-ACM1*-3mut) were grown in selective raffinose medium, and 10-fold serial dilutions were spotted and grown on selective medium containing either glucose or galactose. WT Acm1 rescues lethality caused by Cdh1 overexpression, but the 3mut version of Acm1 is unable to fully rescue the lethality.

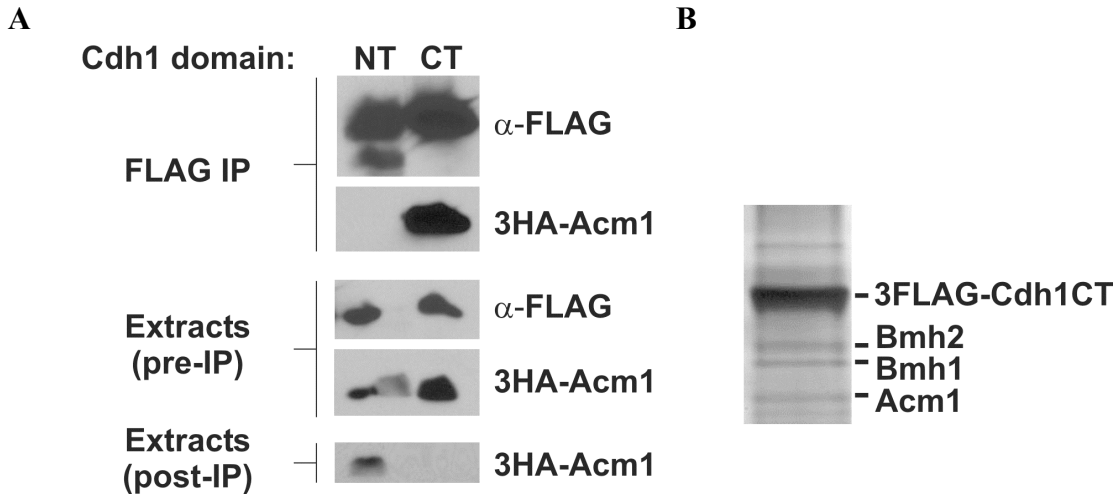


FIGURE 2.3

Figure 2.3: Acm1 binds the WD-40 domain region of Cdh1.
A, Plasmids containing either the N-terminal or C-terminal fragment of Cdh1 were transformed into YKA245 expressing *3HA-ACM1* and co-immunoprecipitated with Cdh1. The N-terminal region of Cdh1 (1-249) contains the prominent CDK phosphorylation sites while the C-terminus (241-end) houses the WD-40 domains. Acm1 interacts with only the C-terminal domain of Cdh1. **B,** The C-terminus of Cdh1 is capable of pulling down the entire Acm1/Bmh1/Bmh2 complex.

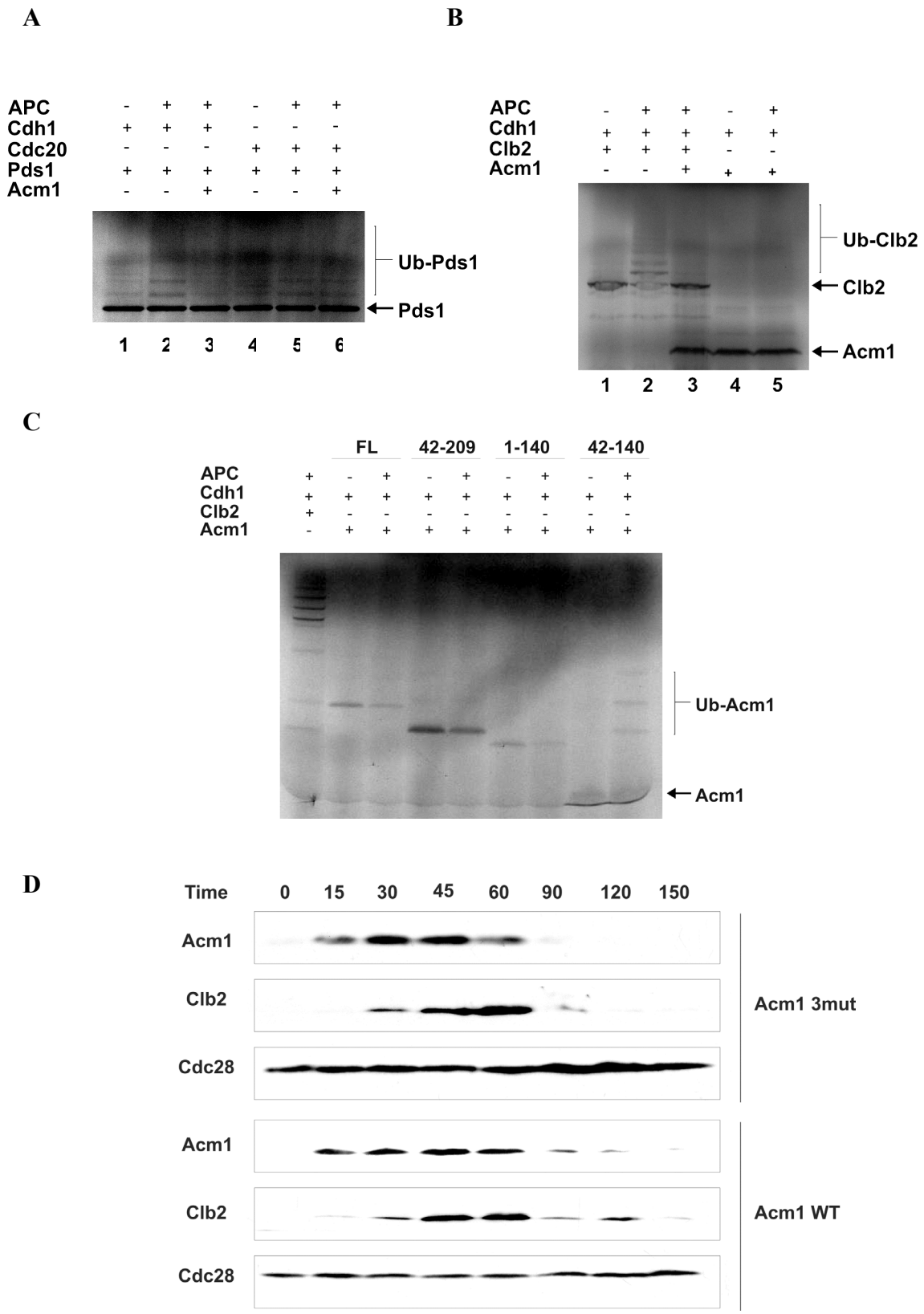

A
APC - + + - + +
Cdh1 + + + - - -
Cdc20 - - - + + +
Pds1 + + + + + +
Acm1 - - + - - +
Ub-Pds1
Pds1
1 2 3 4 5 6
B
APC - + + - +
Cdh1 + + + + +
Clb2 + + + - -
Acm1 - - + + +
Ub-Clb2
Clb2
Acm1
1 2 3 4 5
C
FL 42-209 1-140 42-140
APC + - + - + - + - +
Cdh1 + + + + + + + + +
Clb2 + - - - - - - - -
Acm1 - + + + + + + + +
Ub-Acm1
Acm1
D
Time 0 15 30 45 60 90 120 150
Acm1
Clb2
Cdc28
Acm1 3mut
Acm1
Clb2
Cdc28
Acm1 WT

FIGURE 2.4

Figure 2.4: Acm1 is a specific APC^{Cdh1} inhibitor and is not degraded in an APC dependent manner.
A, *In vitro* ubiquitination assay using recombinant Acm1 for inhibition. Cdh1, Cdc20, Ubc4, and Pds1 used in this assay were produced in the TNT Quick coupled *in vitro* transcription/translation kit. Pds1 was labeled with S^{35} methionine. APC was purified from yeast extracts as described. Ubiquitinated Pds1 can be seen as bands of decreasing gel mobility. Levels of Acm1 5X greater than required for complete APC^{Cdh1} inhibition were not sufficient for inhibition of APC^{Cdc20}. **B,** *In vitro* ubiquitination assay using Cdh1, Ubc4, Clb2, and Acm1 produced in the TNT Quick coupled *in vitro* transcription/translation kit. Clb2 and Acm1 were both labeled with S^{35} methionine. APC was purified from yeast extracts as described. Ubiquitinated Clb2 can be seen as bands of decreasing gel mobility. Full length Acm1 made in the IVTX system can inhibit APC^{Cdh1} activity but is not ubiquitinated by Cdh1-dependent APC. **C,** *In vitro* ubiquitination assay using Cdh1, Ubc4, Clb2, and Acm1 produced in the TNT Quick coupled *in vitro* transcription/translation kit. Truncations of Acm1 were made and designated based on the numbers of amino acids in the sequence. Full length (FL) Acm1 includes amino acids 1-209. The N-terminal truncation of Acm1 is designated 42-209. The C-terminal truncation of Acm1 is designated 1-140 and Acm1 with both termini truncated is designated 42-140. Clb2 and Acm1 were both labeled with S^{35} methionine. APC was purified from yeast extracts as described. Ubiquitinated Clb2 and Acm1 can be seen as bands of decreasing gel mobility. Acm1 with both termini truncated (42-140) can be ubiquitinated by APC^{Cdh1}. **D,** G_1 phase block and release experiment following levels of wild-type Acm1 and the Acm1 mutant (1^{st} and 3^{rd} D box and KEN box mutated) throughout the cell cycle. Cells were synchronized with nocodazole, released, and Acm1 levels measured after the release. No change between Acm1 levels between WT and the Acm1 mutant are observed suggesting Acm1 levels are not controlled by APC activity.

CHAPTER 4

General Discussion

Regulation of the anaphase-promoting complex has been shown to be crucial to maintaining strict cell cycle control ensuring proper progression. Misregulation of the APC co-activators, Cdc20 and Cdh1, has been linked to various cancers. For example, upregulation of Cdc20 has been shown in lung and gastric cancers while downregulation of Cdh1 has been observed in the malignant progression of a B-lymphoma cell lines (75,79). Hence, the study of APC regulation through the co-activators is important for better understanding the cellular processes involved to ultimately design therapeutics to treat cancer.

At the inception of this work, regulation of the APC co-activator Cdh1 in budding yeast was known to occur in several ways. The first and most characterized means of shutting off APC^{Cdh1} activity is through Cdh1 phosphorylation by the cyclin dependent kinase Cdc28. Phosphorylation is thought to disassociate Cdh1 from the APC thereby inactivating APC activity. Other means of regulation include the cell cycle mediated relocalization of Cdh1 during S phase from the nucleus to the cytoplasm. This mechanism is in place presumably to remove Cdh1 from the vicinity of the APC, which is always nuclear. Additional mechanisms for Cdh1 negative regulation exist in other species but before this work, no protein inhibitors of APC^{Cdh1} activity had been identified in budding yeast. The work reported in the previous chapters encompasses the journey from identification to characterization of function to the detailed mechanism of APC^{Cdh1} inhibition by Acm1.

The identification of interacting proteins with the APC co-activator Cdh1 began as a proteomic study of novel APC associated proteins using highly sensitive mass spectrometric techniques. At our disposal was an arsenal of mass spectrometers capable

of analyzing very low-level proteins, ideal for identifying potentially undiscovered interacting proteins of one of the most studied regulatory machines in the cell cycle. As a result, three novel interacting proteins of Cdh1 were identified. Two of the identified proteins were members of the 14-3-3 family of proteins known as Bmh1 and Bmh2. 14-3-3 proteins have been linked to a multitude of cellular processes including relocalization of proteins from the nucleus to the cytoplasm. The third protein identified that interacted with Cdh1, Acm1, was an uncharacterized protein whose identity was linked to Cdc28 as a potential substrate. Our analysis of these Cdh1 interacting proteins revealed an important function for the negative regulation of APC activity at a specific time during the cell cycle.

The interaction between Bmh1, Bmh2, and Cdh1 was found to be dependent on the presence of Acm1. We found Bmh1 and Bmh2 levels to be constant throughout the cell cycle whereas Acm1 levels oscillate with the highest levels occurring in S phase. Hence, complex formation was restricted to the period of the cell cycle during which Acm1 was expressed.

Cells were viable upon deleting Acm1 but exhibited a small perturbation during S phase when Acm1 levels are normally highest. Levels of the APC substrate Clb2 were also slightly lower in *acm1Δ* cells under certain growth conditions. Although these results suggested Acm1 played some role during S phase, the function of Acm1 was not clear until we discovered Acm1 was able to inhibit APC^{Cdh1} activity both *in vitro* and *in vivo*. The addition of Acm1 inhibited APC^{Cdh1} mediated ubiquitination of Clb2 in an *in vitro* activity assay. Overexpression of Acm1 in *sic1Δ* cells was lethal yielding the same

result as a *cdh1Δ sic1Δ* double mutant (112). Overexpression of Acm1 also rescued lethality caused by overexpression of Cdh1.

Although Cdh1 that is heavily phosphorylated cannot activate the APC, partially phosphorylated Cdh1 does have the ability to still activate the E3 ligase. We found Acm1 to inhibit both nonphosphorylated and phosphorylated Cdh1 pointing towards an overlapping Cdh1 inhibition mechanism. Therefore, Acm1 may be in place to complete Cdh1 inactivation when phosphorylation of the co-activator is not sufficient.

We found Acm1 to also be phosphorylated in the cell. Further investigation demonstrated that even though Acm1 is heavily phosphorylated, this modification is not required for functional inhibition. Why then is Acm1 phosphorylated? We observed a slight upshift in the band corresponding to Acm1 on a gel during cell cycle progression when Acm1 levels are highest. A downshift in the Acm1 band is observed prior to its disappearance. Additionally, our collaborator, Mark Hall has observed the instability of Acm1 CDK site phosphomutants mimicking nonphosphorylated Acm1 (Personal communication). It appears phosphorylation of Acm1 may be involved somehow in its stability but at this time, we cannot say how. Additional questions are then raised, like when and by what kinase is Acm1 phosphorylated? What phosphatase removes phosphates from Acm1? Further studies are needed to answer these important questions.

We also found that Bmh1 and Bmh2 were not needed for APC^{Cdh1} inhibition by Acm1 despite them forming a complex with Acm1. The interaction between Bmh1 and Bmh2 was dependent on Acm1 phosphorylation. Since nonphosphorylatable Acm1 is not stable, one cannot help but think one possible role for these proteins may be to aid in Acm1 stabilization. Future directions include dissecting the role of the 14-3-3 proteins in

the complex with Cdh1 and Acm1 to determine if they are involved in protecting Acm1 from premature degradation.

When we first discovered Bmh1 and Bmh2 in complex with Acm1 and Cdh1, it was proposed that one possible role for the 14-3-3 proteins was in the relocalization of Cdh1. Relocalization of Cdh1 from the nucleus to the cytoplasm was shown as another inhibitory mechanism to remove Cdh1 from the vicinity of APC when APC^{Cdh1} activity is not needed (73). Additionally, 14-3-3 proteins were known to be involved in the nuclear/cytoplasmic localization of cell cycle regulatory proteins (102). Therefore, one hypothesis was that Bmh1 and Bmh2 played a similar role with Cdh1. However, upon deleting Acm1, which mediates 14-3-3 binding to Cdh1, no difference in Cdh1 localization between the nucleus and cytoplasm was observed (112). Mark Hall's group found that the complex did prevent Cdh1 localization to the bud neck in yeast (112). The Cdh1 substrates Clb2 and Hsl1 have been shown to localize to the bud neck and these interactions are enhanced in the absence of Acm1 (112,116,117). Since Bmh1 and Bmh2 do not appear to play a direct role in APC^{Cdh1} inhibition, these proteins may serve other important functions *in vivo*. One possibility could be that Acm1/Bmh1/Bmh2 prevent Cdh1 from localizing to the bud neck when Cdh1 is in the cytoplasm. However, further investigation is required to answer this question.

We have also provided evidence that Acm1 acts as an APC^{Cdh1} inhibitor by blocking substrate binding to the co-activator by mimicking an APC substrate. Acm1 contains APC substrate-like D boxes and a KEN box that is involved in substrate recognition and binding. Mutation of these regions of Acm1 abolishes the interaction with Cdh1. Additionally, Cdh1 contains WD-40 domains in its C-terminus that are

implicated in substrate binding. We have shown Acm1 binds to the C-terminus of Cdh1. From gel filtration experiments, it appears Acm1 interacts with Cdh1 in a 1:1 ratio. This suggests that Acm1 binds to a single site on Cdh1. If Acm1 binds to a single Cdh1 site and the addition of Acm1 decreases substrate binding to Cdh1, then Acm1 must be a competitive inhibitor.

Although Acm1 looks like an APC substrate, full length Acm1 does not appear to be ubiquitinated by the APC. However, regions of Acm1 appear to prevent it from ubiquitination by the Cdh1-dependent APC. Truncations of the N-terminus and C-terminus of Acm1 were used in an *in vitro* ubiquitination assay revealing that both termini protect Acm1 from being processed as a substrate. Without the intact N-terminus and C-terminus, Acm1 is ubiquitinated by APC^{Cdh1}. Perhaps these regions block lysine residues from ubiquitination allowing Acm1 to act as a potent inhibitor by competing for substrate binding. Since the SCF complex is the other main E3 ligase involved in cell cycle dependent proteolysis of proteins, it would be easy to suggest the SCF may play a role in the degradation of Acm1. However, there is no precedent for SCF-mediated degradation of proteins in late Mitosis or that phosphorylation protects substrates from SCF targeting. Furthermore, we do not know at this point if Acm1 is subjected to a ubiquitin-dependent proteolytic event. Additional experimentation is needed to determine if Acm1 is degraded in a proteasome dependent manner and what machinery is potentially involved.

The discovery of Acm1, Bmh1, and Bmh2 in complex with Cdh1 has provided us with additional insight into APC regulation. APC regulation is of the utmost importance for maintaining strict cell cycle control thereby preventing genetic catastrophe. Because

of this importance, multiple, overlapping mechanisms are in place to ensure complete inactivation of the APC for proper cell cycle progression. The results illustrated in this dissertation may help in our global understanding of APC regulation leading to better comprehension of healthy cellular processes for the eventual prevention and treatment of diseases in the future.

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