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Direct Binding of CDC20 Protein Family Members Activates the Anaphase-Promoting Complex in Mitosis and G1

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Summary

Activation of the anaphase-promoting complex (APC) is required for anaphase initiation and for exit from mitosis. We show that APC is activated during mitosis and G1 by two regulatory factors, hCDC20 and hCDH1. These proteins directly bind to APC and activate its cyclin ubiquitination activity. hCDC20 confers a strict destruction-box (D-box) dependence on APC, while hCDH1 shows a much more relaxed specificity for the D-box. In HeLa cells, the protein levels of hCDC20 as well as its binding to APC peak in mitosis and decrease drastically at early G1. Thus, hCDC20 is the mitotic activator of APC and directs the degradation of substrates containing the D-box. The hCDH1 protein level remains constant during the cell cycle and may target specific substrates lacking the D-box in G1, such as polo-like kinase, for ubiquitination.

Introduction

It has only been recently appreciated that general mechanisms for protein degradation can regulate critical cellular events in the cell cycle, signal transduction, and development (Glotzer et al., 1991; Holloway et al., 1993; Jiang and Struhl, 1998). In the case of mitotic control, cyclin B is degraded by the ubiquitin-mediated pathway to allow cells to exit mitosis and enter the subsequent G1 state (Glotzer et al., 1991). As more has been learned about the degradation steps at mitosis, it is clear that they are under complex and specific control. Cyclin degradation is regulated by the activation of the cdc2/cyclin B complex (Hershko et al., 1994; King et al., 1995; Lahav-Baratz et al., 1995; Peters et al., 1996). Yet, time must be set aside between the activation of the kinase and the onset of proteolysis for the nucleus of animal cells to disassemble, the spindle to form, and the chromosomes to condense and align properly at the metaphase plate. In cases of spindle damage, this process is temporarily arrested to allow for repair (Elledge, 1996; Rudner and Murray, 1996). More detailed investigations of mitosis suggested that degradation is indeed the key process that initiates anaphase but that cyclin degradation occurs much later at the end of anaphase. Other substrates are degraded at the onset of anaphase (Holloway et al., 1993). Therefore, the proteolysis pathway in the mitotic cycle must be under complex controls, regulating time, substrate specificity, and perhaps location.

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Biochemical and genetic investigations have shown that the proteolysis events in anaphase and telophase are regulated by the last component of the ubiquitin conjugation pathway, an ubiquitin protein ligase of 1500 kDa known as the anaphase-promoting complex (APC) (Irniger et al., 1995; King et al., 1995; Sudakin et al., 1995; Tugendreich et al., 1995). This complex recognizes a 9 amino acid sequence (destruction box [D-box]) in the N terminus of cyclin and a few other proteins, catalyzing the transfer of ubiquitin from the thioester of a UBC–ubiquitin complex to a free amino group on the protein and then linking further ubiquitins to the ubiquitinated substrate to generate a large chain of ubiquitins; the tagged protein is then recognized by the proteasome and degraded (Glotzer et al., 1991; King et al., 1995; Aristarkhov et al., 1996; King et al., 1996; Yu et al., 1996). Recently, sister chromatid separation at the onset of anaphase has been shown to be regulated by the APC-mediated destruction of anaphase inhibitors, Pds1p in *Saccharomyces cerevisiae* (*S. cerevisiae*) and Cut2p in *Schizosaccharomyces pombe* (*S. pombe*) (Cohen-Fix et al., 1996; Funabiki et al., 1996; Yamamoto et al., 1996). Later, the degradation of the spindle-associated protein Ase1p and mitotic cyclins by the APC pathway is required for exit from mitosis (Glotzer et al., 1991; King et al., 1995; Juang et al., 1997).

The molecular composition of APC is highly conserved in eukaryotic organisms. Vertebrate APC consists of eight subunits, named APC1 to APC8 (King et al., 1995; Peters et al., 1996; Yu et al., 1998). Six of the APC subunits have homologs in budding yeast, and APC has been genetically shown to be involved in regulating the onset of anaphase (Irniger et al., 1995; Zachariae and Nasmyth, 1996; Zachariae et al., 1996, 1998; Yu et al., 1998). APC activity is low in S, G2, and early mitosis. It becomes active in late mitosis to degrade anaphase inhibitors and mitotic cyclins, and its activity seems to persist until late G1 phase in yeast and mammalian cells (Amon et al., 1994; Brandeis and Hunt, 1996). APC appears to be the only cell cycle-regulated component of this degradation pathway (King et al., 1995; Peters et al., 1996; Yu et al., 1996), and hence we might expect that much of the detailed temporal and fidelity control will be focused on APC.

Biochemical studies have suggested that phosphorylation of four APC subunits may regulate APC activity. There is more than enough potential complexity in the phosphorylation sites to account for the complexity of regulation (King et al., 1995; Lahav-Baratz et al., 1995; Peters et al., 1996). However, recent genetic evidence has suggested that other components may play an important role in the regulation of APC activity. Two WD-40 repeat containing proteins, Cdc20p and Cdh1p/Hct1p in *S. cerevisiae* and *fizzy* (*fzy*) and *fizzy-related* (*fzr*) in *Drosophila melanogaster*, have been shown to activate cyclin degradation through APC (Schwab et al., 1997; Sigrist and Lehner, 1997; Visintin et al., 1997). Ectopic expression of Cdc20p or Cdh1p at any point of the yeast cell cycle causes degradation of Clb2p or Pds1p (Schwab et al., 1997; Visintin et al., 1997). Overexpression of *fzr*

in *Drosophila* also lowers the level of mitotic cyclins (Sigrist and Lehner, 1997). It has been proposed that these CDC20-related proteins might act as substrate-specific regulators of the APC pathway (Schwab et al., 1997; Visintin et al., 1997), though it is unclear whether they act directly on APC or its substrates or through some upstream control circuit. These studies have raised the question of whether substrate selection and temporal control acts through these WD-40 repeat proteins, and it therefore seems essential for us to understand how these proteins function, when they function, and what role they might have in substrate selection.

When we began to identify activators of APC by purely biochemical means, we initially fractionated mitotic *Xenopus* egg extracts and found two activities that could independently activate interphase APC. One activity was dependent on ATP and was likely to be a kinase (though clearly not cdc2). The other activity activated APC in the absence of ATP. In this report, we identify the ATP-independent activator as the vertebrate homolog of yeast Cdc20p. We have also cloned the human homolog of yeast CDH1/HCT1, hCDH1 (human CDH1). Correspondingly, we propose to rename the human homolog of CDC20 (p55CDC) as hCDC20 (human CDC20) (Weinstein et al., 1994). We show that these WD-40 repeat proteins activate APC by directly binding to it. These proteins also affect substrate selection with the CDC20 protein showing strict requirement for the 9 amino acid D-box for recognition, while the CDH1 protein has a relaxed specificity for D-box. It is very likely that the synthesis, binding, and degradation of these proteins control much of the temporal specificity of the post-anaphase cell cycle. The level of hCDC20 protein peaks at G2/M and drops sharply as cells exit mitosis, consistent with its role as the mitotic activator of APC. In contrast, the protein level of hCDH1 does not vary during the cell cycle and would appear to be the sole G1 regulator of APC, although it may also be involved in the regulation of some of the late mitotic activity of APC.

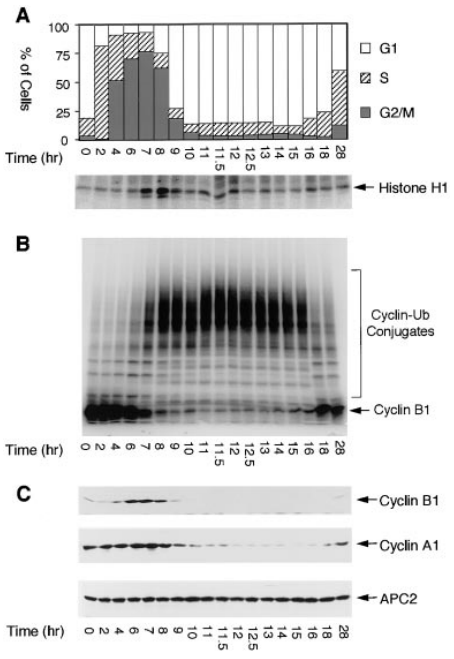

Figure 1. Cell Cycle Regulation of the APC Activity

HeLa cells were synchronized at the G1/S boundary by a double thymidine block, and cells were collected at indicated timepoints after being released from the arrest.

(A) Cell cycle stage was determined by FACS analysis. Cdc2 kinase was immunopurified from cell lysates and assayed for histone H1 kinase activity.

(B) APC was purified from the cell lysates with anti-CDC27/protein A beads and analyzed for its ability to ubiquitinate a ^{125}I-labeled N-terminal fragment of *Xenopus* cyclin B1. CDC27 is a subunit of APC.

(C) Levels of cyclin B1, cyclin A1, and APC2 were determined by immunoblotting.

Results

The Activity of APC in the HeLa Cell Cycle

Using the N-terminal fragment of *Xenopus laevis* cyclin B1 as a substrate, we analyzed the activity of the cyclin ubiquitinating activity of APC during the HeLa cell cycle. HeLa cells were synchronized by a double thymidine block, released from their G1/S arrest, and sampled for 28 hr, during which time they completed S phase, G2, mitosis, and entered the next S phase (Figure 1A). APC was immunopurified from each sample and assayed. APC becomes fully activated late in mitosis, remains active through most of G1, and is rapidly inactivated at the G1/S transition, as judged by the quantitative conversion of the cyclin B1 fragment to high molecular mass ubiquitin conjugates (Figure 1B). The activity of immunopurified APC in G1 is consistent with previous findings in yeast and mammalian cells that APC substrates continue to be degraded in G1 (Amon et al., 1994; Brandeis and Hunt, 1996). The activation of APC in mitosis coincides roughly with the onset of cyclin B1 and A1 degradation (Figure 1C, 8 hr). The level of APC subunits remains constant throughout the cell cycle (Figure 1C; data not shown). Hence, onset and loss of APC activity is not related to the abundance of APC.

Factors in Mitotic *Xenopus* Extracts that Activate Interphase APC

To identify factors that could regulate the activity of APC, mitotic *Xenopus* egg extracts were fractionated by anion exchange chromatography and mixed with inactive interphase APC immunopurified from interphase egg extracts. The immunopurified interphase APC possesses the characteristic eight APC subunit bands and contains no appreciable contaminating proteins, as judged by electrophoresis and silver staining (data not shown) (Peters et al., 1996). After incubation with fractionated mitotic extracts, the interphase APC beads were washed with buffer and assayed for cyclin B ubiquitination activity. Fractions 5–14 contained at least 3 activities with peaks in 5–7, 8–10, and 11–14 that activated APC in the presence of ATP (Figure 2A). However, in the absence of ATP, the activity in fractions 8–10 disappeared (labeled peak B in Figure 2B), leaving at least two peaks. The activity in peak C (11–14) cofractionated with mitotic APC as assayed by immunoblotting with an anti-APC antibody (data not shown) and is likely

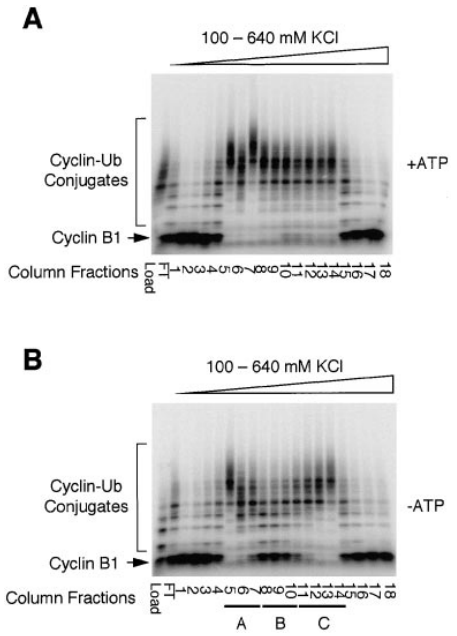

Figure 2. Identification of an ATP-Dependent and an ATP-Independent Activator of APC from the Mitotic *Xenopus* Extracts

Mitotic *Xenopus* egg extracts were fractionated through a Resource Q column, and bound proteins were eluted with a gradient of 100–640 mM KCl. Column fractions were desalted, concentrated, and assayed for activation of the *Xenopus* interphase APC either in the presence of ATP (A) or in the absence of ATP (B).

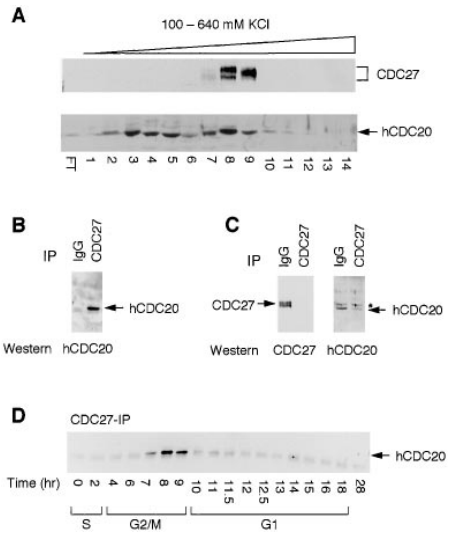

Figure 3. Mitotic Activators of APC in HeLa Cell Extracts and Association of Mitotic APC with hCDC20

(A) Mitotic extracts from synchronized HeLa cells were fractionated through a Resource Q column, and bound proteins were eluted with a gradient of 100–640 mM KCl. Fractionation profiles of CDC27 and hCDC20 were determined by immunoblotting.

(B and C) APC-containing fractions (7–9) were pooled and subjected to immunoprecipitation by either anti-CDC27 antibody beads or control rabbit IgG beads. The immunoprecipitates (B) and the supernatants after immunoprecipitation (C) were blotted with anti-CDC27 and anti-hCDC20 antibodies. The cross-reacting band detected by the anti-hCDC20 antibody in (C) was marked by a star and served as a loading control.

(D) APC was purified from synchronized HeLa cell lysate (see Figure 1A for FACS analysis) by anti-CDC27 antibody beads, and association of hCDC20 with APC was determined by immunoblotting with anti-hCDC20 antibody.

to reflect the binding of active mitotic APC to the antibody beads. Thus, there is one ATP-independent activity (peak A) and one ATP-dependent activity (peak B) present in the mitotic extracts. The ATP-dependent activity is likely to contain a kinase. However, cdc2 does not appear to be the kinase that activates APC, since it separates from the activity upon further fractionation with a hydrophobic interaction column; furthermore, immunodepletion of cdc2 from the activating fraction did not affect its ability to activate APC in the presence of ATP (data not shown).

The ATP-independent fractions eluted on a gel filtration column with a molecular weight of 60 kDa (data not shown). Because the CDC20-related proteins of that mass, possessing no kinase activity, have been implicated genetically in APC regulation, we investigated whether homologs of yeast CDC20 and *Drosophila fizzy* were responsible for the observed activation of APC. We used HeLa cell extracts due to the availability of an antibody against the human CDC20 protein (hCDC20). As shown in Figure 3A, some hCDC20 protein cofractionated with APC on anion exchange chromatography. More importantly, in the APC fractions, hCDC20 coimmunoprecipitated with CDC27, a subunit of APC, and anti-CDC27 beads depleted a large portion of hCDC20 (Figures 3B and 3C). Furthermore, we observed an association of hCDC20 with APC in vivo only in late mitosis to early G1 (Figure 3D), which correlated with the time of APC activation during the cell cycle (compare Figures 3D and 1B). These strong physical correlations suggested that the hCDC20 binds to APC and might regulate its activity.

hCDC20 and hCDH1 Bind to and Activate APC

There are two members in the CDC20 protein family: Cdc20p and Cdh1p/Hct1p in yeast, *fizzy* and *fizzy-related* in *Drosophila*. Both members have been implicated in regulating the APC pathway. To study the human homolog of yeast CDH1, we cloned it after searching the human expressed sequence tag (EST) database with the sequence of hCDC20 using the BLAST program. Multiple ESTs were identified that share significant sequence similarity with hCDC20. On the basis of these EST sequences, we isolated a full-length cDNA; its sequence revealed that, like hCDC20, it encodes seven WD40 repeats in its carboxy-terminal region. This gene is more closely related to *CDH1/HCT1* and *fzr* than to *CDC20* and *fzy*. Its gene product shares high amino acid sequence identity with *Xenopus* and *Drosophila fzr* (96% and 69%, respectively) and with *S. cerevisiae* Cdh1p (36%). We therefore name this gene *hCDH1*. The hCDH1 and hCDC20 proteins show more conservation in their WD-40 repeats (49% identity) and share less sequence identity in their amino-terminal regions (19%).

To test whether hCDC20 and hCDH1 can directly activate APC, the proteins were synthesized in reticulocyte lysates and then incubated with inactive interphase *Xenopus* APC, which was immunopurified on anti-CDC27 beads; the beads were washed and assayed for cyclin

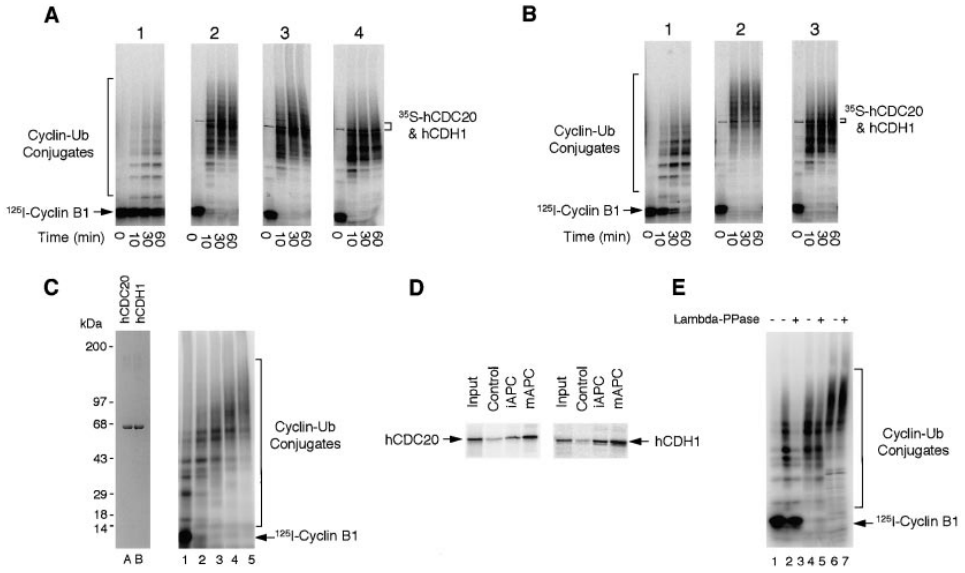

Figure 4. Activation of APC by hCDC20 and hCDH1

(A) ^{35}S-labeled hCDC20 (2), hCDH1 (3), and *Xenopus* CDH1 (4) were translated in reticulocyte lysate and incubated with interphase APC immunopurified from *Xenopus* egg extracts. Incubation of the APC beads with reticulocyte lysate alone (1) was included as a control. The APC beads were then washed and assayed for its ability to ubiquitinate a ^{125}I-labeled N-terminal fragment of *Xenopus* cyclin B1.

(B) Same as (A) except that mitotic APC beads were used.

(C) Activation of APC by purified hCDC20 and hCDH1. HA-tagged hCDC20 and hCDH1 were synthesized in reticulocyte lysates and immunopurified with the anti-HA antibody coupled to the protein A beads. After stringent wash, hCDC20 and hCDH1 bound to the beads were eluted with the HA peptide, separated by SDS-PAGE, and detected by silver staining (lanes A and B). The eluted hCDC20 (lane 2) or hCDH1 (lane 4) was incubated with immunopurified interphase APC. Incubation of interphase APC beads with the eluate from a mock anti-HA immunoprecipitation was included as a negative control (lane 1), and incubation of unpurified hCDC20 (lane 3) or hCDH1 (lane 5) in reticulocyte lysates was included as positive controls. The beads were then washed and assayed for their ability to ubiquitinate a ^{125}I-labeled N-terminal fragment of *Xenopus* cyclin B1.

(D) ^{35}S-labeled hCDC20 and hCDH1 were incubated with either interphase (i) or mitotic (m) APC immunopurified on anti-CDC27 antibody beads. After being washed five times with the Q-A buffer containing 500 mM KCl and 0.5% NP-40, hCDC20 and hCDH1 retained on beads were analyzed by SDS-PAGE. Anti-CDC27 antibody beads alone were used as controls. Input lanes contain 10% of hCDC20 and hCDH1 proteins added to reactions.

(E) Mitotic APC (lanes 2 and 3), hCDC20-activated APC (lanes 4 and 5), and hCDH1-activated APC (lanes 6 and 7) were incubated either with λ-phosphatase (lane 3, 5, and 7) or with buffer (lanes 2, 4, and 6) and then assayed for cyclin ubiquitination. Lane 1, untreated interphase APC for basal activity.

B ubiquitination activity. Incubation of APC beads with reticulocyte lysates alone was included as a control (Figure 4A). Both hCDC20 and hCDH1 activated interphase APC extremely efficiently. Within 10 min, about 90% of the substrate was converted to ubiquitin conjugates by the activated APC, while only 25% of the cyclin B substrate was conjugated to ubiquitin by interphase APC. Furthermore, the conjugates formed by the activated APC were of much higher mass (Figure 4A). In vitro translated *Xenopus* CDH1 activated *Xenopus* interphase APC with a similar efficiency as hCDH1 (Figure 4A, gel 4), demonstrating that the mechanism of APC activation is conserved during evolution and justifying our use of the human homologs with *Xenopus* interphase APC. The cyclin ubiquitination activity of the activated APC was greater than that of mitotic APC, both in terms of reaction kinetics and in terms of the average molecular mass of cyclin–ubiquitin conjugates formed (cf. Figure 4A, gels 2 and 3, versus Figure 4B, gel 1). Consistent with this finding, incubation of mitotic APC with hCDC20 or hCDH1 also enhanced its ubiquitination activity (Figure 4B), suggesting that mitotic APC contains substoichiometric amounts of these activators and that hCDC20 and hCDH1 are limiting factors in the activation of APC.

We were unable to obtain soluble forms of the hCDC20 or hCDH1 proteins in bacteria. To rule out the possibility that factors in the reticulocyte might be required along with hCDC20 and hCDH1 to activate APC, we purified HA-tagged hCDC20 and hCDH1 proteins from the reticulocyte lysates by precipitating these proteins with anti-HA beads and eluting the bound proteins with an HA peptide. As shown in Figure 4C (lanes A and B), both hCDC20 and hCDH1 were purified to apparent homogeneity, as judged by electrophoresis and silver-staining. The eluted proteins were then analyzed in the activation assay as described above. Both purified hCDC20 and hCDH1 proteins activated interphase APC efficiently, both in terms of the percentage of the substrate converting to ubiquitin conjugates and in terms of the average molecular weight of the conjugates formed (Figure 4C, lanes 2 and 4). Thus, hCDC20 and hCDH1 are sufficient for activation.

To demonstrate a direct interaction between APC and hCDC20/hCDH1, interphase or mitotic APC purified on antibody beads were incubated with ^{35}S-labeled hCDC20 or hCDH1 proteins and then washed stringently. As shown in Figure 4D, both proteins selectively interacted with interphase and mitotic APC, but both

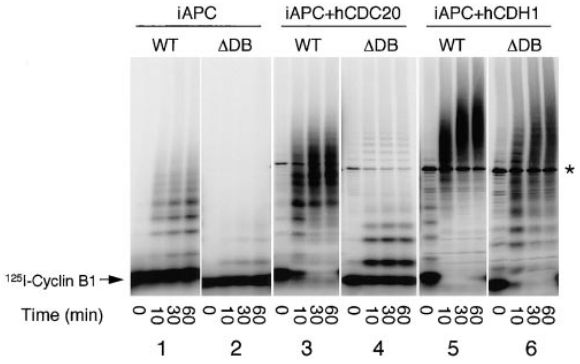

Figure 5. D-Box Specificity of hCDC20 and hCDH1 Activation

Assays were performed as described in Figure 4A except that a ^{125}I-labeled D-box deletion mutant of cyclin B1 was used in 2, 4, and 6. The APC-bound ^{35}S-labeled hCDC20 and hCDH1 are labeled with an asterisk.

appeared to have higher affinities for mitotic APC than for interphase APC. Therefore, hCDC20 and hCDH1 activate APC in vitro and bind to APC both in vivo and in vitro.

These binding studies suggest that phosphorylation may affect the affinity of APC to its regulatory subunits but that phosphorylation is not absolutely required for some degree of interaction. Dephosphorylation of mitotic APC by λ-phosphatase had previously been shown to inactivate mitotic APC (Peters et al., 1996). To test further the role of phosphorylation in APC activation, APC was first incubated with hCDC20 or hCDH1 in the presence or absence of λ-phosphatase and assayed for cyclin ubiquitination activity. Consistent with previous results, λ-phosphatase treatment inactivated mitotic APC (Figure 4E, lanes 2 and 3). However, addition of λ-phosphatase during the incubation of hCDC20 or hCDH1 with APC had no effect on their ability to activate APC (Figure 4E, lanes 4–7). Similarly, when the mitotic APC was first dephosphorylated with λ-phosphatase and then incubated with hCDC20 and hCDH1, APC was still fully activated (data not shown). These experiments indicate that the critical step in the activation of APC is binding of hCDC20 and hCDH1, not phosphorylation of APC. More complete studies of the affinity of hCDC20 and hCDH1 for APC will be required to assess the in vivo role of phosphorylation, which may occur under different buffer conditions or where both activators are present at much lower concentrations.

hCDC20 and hCDH1 Differ in D-Box Specificity

In addition to regulating APC temporally, the WD-40 proteins, hCDC20 and hCDH1, could affect substrate specificity. Little is known about substrate selection except that all APC substrates identified so far contain a destruction-box (Glotzer et al., 1991). APC activated by either hCDC20 and hCDH1 ubiquitinates the N-terminal fragment of cyclin B1 efficiently; however, they have different D-box requirements. hCDC20-associated APC is strictly D-box-specific, with the wild-type cyclin B fragment much more efficiently ubiquitinated than the D-box deletion mutant (Figure 5). In contrast, APC activated by hCDH1 supported the ubiquitination of both the wild-type and the D-box deletion mutant of cyclin B, although the wild-type substrate formed ubiquitin conjugates faster and with a greater average molecular weight than the mutant (Figure 5A). Substrate competition experiments also indicate that the hCDC20-activated APC strictly requires D-box for substrate recognition, while the hCDH1-associated APC has a much less stringent requirement for D-box (data not shown). Though hCDH1-associated APC does not absolutely require D-box for substrate recognition, neither was it permissive in ubiquitination. When we analyzed more than twenty individual proteins translated from random cDNA clones as well as five groups of proteins each synthesized from pools of 30 cDNAs, we found that none was a substrate of hCDH1-activated APC (data not shown). Thus, our data suggest that hCDC20 confers D-box specificity of APC, whereas APC activated by hCDH1 can recognize the N-terminal fragment of cyclin B1 lacking the D-box. It is therefore possible that APC activated by hCDH1 may ubiquitinate substrates that do not contain the D-box; these substrates presumably share other structural features.

The Levels of hCDC20 and hCDH1 Are Regulated Differently during the Cell Cycle

Some features of the activity of APC toward specific substrates in the cell cycle might be determined by the expression of hCDC20 and hCDH1. The mRNA levels of both hCDC20 and hCDH1 accumulate in G2, persist through mitosis, and disappear in early G1 (Figure 6A), as determined by Northern blots from synchronized HeLa cells. Despite the oscillation in the levels of *hCDH1* mRNA, the protein level of hCDH1 does not vary appreciably through the cell cycle (Figure 6B). We obtained the same result using two different affinity-purified anti-hCDH1 antibodies made against different epitopes on hCDH1. Thus, hCDH1 must be a stable protein whose transcriptional periodicity has little effect on its protein level. In contrast, the level of hCDC20 protein follows the mRNA pattern, increasing from late G2 through mitosis and dropping drastically in early G1 (Figure 6B). Thus, the hCDC20 protein is most likely unstable.

The presence of APC activity with hCDH1 in G1 suggests that APC may target substrates lacking D-boxes. The polo-like kinase (Plk1), a kinase required for spindle duplication and entry into mitosis, varies during the cell cycle (Figure 6C) (Lee and Erikson, 1997). Plk1 protein is absent in S phase, begins to accumulate in G2, and peaks in mitosis. This kinase is stable throughout mitosis and only disappears in early G1, suggesting that it might be a substrate of APC. Indeed, Plk1 is a substrate of both the hCDC20- and hCDH1-activated APC (Figure 6D), although it is much more efficiently ubiquitinated by the hCDH1-activated APC (Figure 6D). This is consistent with the fact that Plk1 lacks a well-defined D-box.

Discussion

A model for the cell cycle regulation of APC is shown in Figure 7 with evidence and discussion presented below. APC is phosphorylated in mitosis by kinases under the

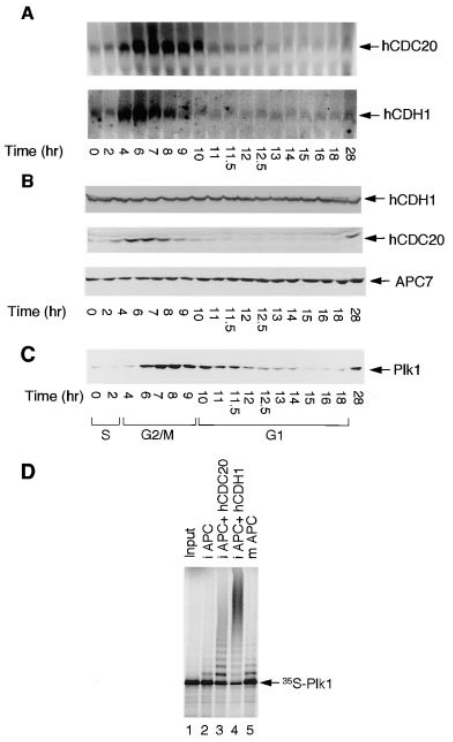

Figure 6. Cell Cycle Regulation of hCDC20 and hCDH1 Expression

(A) Total RNA was purified from synchronized HeLa cells (see Figure 1A), and the expression level of hCDC20 and hCDH1 was determined by Northern blot analysis with random primed ^{32}P-labeled probes corresponding to the complete coding regions of these two genes.

(B) Lysates from synchronized HeLa cells (see Figure 1A) were blotted with anti-hCDH1, hCDC20, and APC7 antibodies.

(C) Levels of Plk1 in the synchronized HeLa cells (Figure 1A) were determined by immunoblotting.

(D) Plk1 as a substrate of APC. Nonradioactive hCDC20 (lane 3) and hCDH1 (lane 4) were incubated with interphase APC beads. The activated APC beads, along with interphase (lane 2) or mitotic (lane 5) APC beads, were incubated with ^{35}S-labeled human Plk1 in the presence of E1 and UBCx, and ubiquitin conjugates were analyzed by SDS-PAGE. Input (lane 1) contains the same amount of Plk1 as added to the ubiquitination reactions.

regulation of cdc2. Phosphorylation increases the affinity of APC toward CDC20 and CDH1. The binding of these proteins to the phosphorylated form of APC leads to its complete activation. Active APC initiates chromosome separation and more slowly leads to mitotic cyclin degradation and spindle remodeling. As cells exit from mitosis, APC is dephosphorylated due to the loss of cdc2 activity, and dephosphorylation of APC may cause the release of CDC20. Ultimately, some event at the G1/S boundary (perhaps mediated by cyclin E/cdk2) inactivates APC, possibly by causing the release or modification of CDH1.

Mechanism of APC Activation

APC is known to be phosphorylated during mitosis, and evidence has been presented here again to support previous reports that phosphorylation of APC enhances its ubiquitination activity. However, the role of phosphorylation in the activation of APC is rather complex and will require further quantitative studies. Dephosphorylation

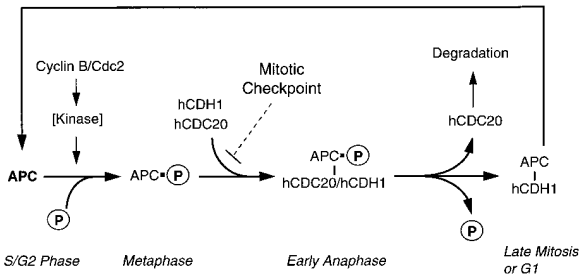

Figure 7. Model for the Cell Cycle Regulation of APC

See text for further details.

by λ-phosphatase did not affect the activation of either interphase or mitotic APC by either hCDC20 or hCDH1, when they were present at saturating levels. Furthermore, although incubation of mitotic APC with λ-phosphatase reduced its activity to basal level, binding of hCDC20 or hCDH1 to the λ-phosphatase-treated mitotic APC reactivated its activity (data not shown). Thus, our data suggest that binding of CDC20 and CDH1, not phosphorylation of APC subunits, is the critical step in activation of APC. This explains the observation that overexpression of Cdc20p or Cdh1p/Hct1p in yeast causes degradation of APC substrates at any stage of the cell cycle (Schwab et al., 1997; Visintin et al., 1997).

Phosphorylation is likely to make a critical contribution to activation of APC in vivo. The hCDC20 and hCDH1 proteins seem to bind to the phosphorylated form of APC with higher affinity than to the interphase APC (Figure 4D), although a firm conclusion on binding affinity awaits quantitative measurement of binding constants and better characterization of the phosphorylation state of APC. We also found that a kinase in mitotic extracts can activate interphase APC, presumably by phosphorylating APC and enhancing its association with the small amount of CDC20 present in these fractions. Since CDC20 and CDH1 appear to be the limiting factors in activation of APC, and mitotic APC only contains substoichiometric amounts of these proteins (data not shown), this difference in affinity could also explain why λ-phosphatase treatment inactivates mitotic APC but does not have any effect on the activation of APC when exposed to an excess amount of CDC20 or CDH1. We speculate that at limiting amounts of CDC20 or CDH1, dephosphorylation of mitotic APC by the λ-phosphatase may cause dissociation of the bound activator and therefore inactivation of mitotic APC, although the direct test of this hypothesis awaits availability of antibodies against the *Xenopus* CDC20 and CDH1. In addition to the phosphorylation on APC, both hCDC20 and hCDH1 are themselves phosphorylated during mitosis (data not shown), and the physiological function of these phosphorylations remains to be established.

How does binding of hCDC20 and hCDH1 activate APC? One possibility is that these two proteins act as accessory proteins bridging substrates to APC. We were unable to detect any association between these proteins and mitotic cyclins (A1 and B1) in immunoprecipitation experiments (data not shown). This is not surprising, since interactions between enzymes and substrates are

usually weak. In contrast, CDC20 or CDH1 forms a stable complex with APC. Because CDC20- and CDH1-associated APC have different requirements of D-box for substrate recognition, we may speculate that these proteins are involved in binding substrates to APC. In certain cases, ubiquitin-protein ligases seem to catalyze the ubiquitination of their substrates through the formation of ligase-ubiquitin thioesters (Scheffner et al., 1995). It is possible that APC may also utilize a similar mechanism in substrate ubiquitination, though we have not been able to detect such a thioester. We noticed a cysteine residue in the WD-40 repeats that is conserved among all seven CDC20/CDH1 proteins identified so far. However, the conserved cysteine residue of CDC20 proteins is unlikely to play a role in catalysis because it seems to be buried in a hydrophobic core, based on the structure modeling of the CDC20 WD-40 domain with the X-ray structure of β-transducin WD-40 domain as a template (data not shown) (Lambright et al., 1996).

Regulation of APC Activation during Mitosis

Expression of CDC20 is controlled on the mRNA level and by protein stability. Although the hCDC20 protein level peaks around 6–8 hr after release from the G1/S block, APC is not activated until 7 hr after release and reaches its maximum activity 8 hr after release (Figure 1B). Consistent with the activity profile, binding of hCDC20 to APC does not occur until 7–8 hr after release (Figure 3D). Furthermore, the association between hCDC20 and APC persists up to 9 hr after release when the overall level of hCDC20 protein has already decreased drastically (cf. Figure 3D versus Figure 6B). Finally, the existence of high levels of hCDH1 protein in S, G2, and early mitosis does not result in premature activation of APC. Thus, in addition to the regulation of hCDC20 and hCDH1 expression, there must be additional mechanisms that control the timing of APC activation.

One pathway that might prevent premature binding of CDC20 to and therefore activation of APC is the spindle assembly checkpoint (Rudner and Murray, 1996). It has been shown that the APC pathway remains inactive when the sister chromatids have not been aligned at the metaphase plate, and the spindle assembly checkpoint is activated. It is conceivable that this checkpoint inhibits the activation of APC and initiation of anaphase by preventing the binding of CDC20 to APC (Figure 7). Consistent with this model, it has recently been shown that CDC20 forms a stable complex with a spindle checkpoint protein MAD2 in both budding and fission yeasts (Fang et al., 1998; Jiang and Struhl, 1998). We have shown that the human MAD2 homolog forms a ternary complex with hCDC20 and APC and prevents premature activation of APC at metaphase (Fang et al., 1998). In addition, this lag may simply correspond to the time needed for the phosphorylation of CDC20, CDH1, or APC. In addition, we cannot exclude the possibility that CDC20 and CDH1 may be negatively regulated by phosphorylation during mitosis, and removal of such inhibitory phosphorylation is required for their function as APC activators. It is also possible that CDC20 and CDH1 may be sequestered by regulatory factors until the time of APC activation.

The Function of APC in G1

APC is active in G1, and CDH1 is likely to be the G1 activator of APC based on the following observations. Like the mitotic activator hCDC20, hCDH1 can bind to and directly activate APC, but, unlike hCDC20 whose level drops after mitosis, the level of hCDH1 protein remains constant throughout the cell cycle. Genetic studies in *Drosophila* showed that *fzr*, a CDH1 homolog, is required for cyclin degradation during G1. Similarly, *srw1*, a CDH1 homolog in *S. pombe*, controls the level of B-type cyclin cdc13 and is required for G1 arrest when cells are starved for nitrogen source (Yamaguchi et al., 1997). Thus, both our biochemical analysis and genetic studies in *Drosophila* and yeast suggest a role of CDH1 in mediating the G1 activity of APC.

A role for CDC20 and CDH1 in substrate selection is suggested by both genetic and our biochemical studies. In yeast, it has been suggested that CDC20 and CDH1 may be substrate-specific activators of the APC pathway: CDC20 for Pds1p and CDH1 for Clb2p and Ase1p. We show here that CDC20 and CDH1 differ in their D-box dependency and show different abundance at various cell cycle phases. The timing of the degradation of APC substrates might be controlled by the composition of APC at different phases of the cell cycle. As cells exit from mitosis, it is possible that CDH1 replaces CDC20 from APC; the free CDC20 is then degraded. Hence, displacement and degradation of CDC20-like proteins may account for some of the detailed timing of APC substrate specificity in late mitosis and early G1. It is not known how the CDH1/APC activity is down-regulated at the G1/S transition. The timing of APC inactivation coincides with the activation of cyclin E/Cdk2, and genetic data in yeast and *Drosophila* have also implicated S phase CDKs in APC inactivation.

What is the function of APC in G1? The data in yeast suggest that APC needs to remain active during G1 to keep mitotic cyclins from accumulating (Amon et al., 1994). In budding yeast, the G1 APC is also required to prevent premature entrance into S phase; inactivation of APC in G1 results in precocious DNA replication (Irniger and Nasmyth, 1997). We also found that the polo-like kinase, Plk1, is a substrate of APC in G1. This suggests that another function of APC in G1 is to inactivate essential mitotic regulators, such as Plk1, to reset the cell cycle. It is also conceivable that there are other cell cycle events mediated by APC in G1. To pursue this, it will be useful to look for substrates of CDH1/APC with relaxed D-box specificity. In particular, the inactivation of APC at the G1/S boundary raises the question whether it is a key regulated event in this transition.

Experimental Procedures

Antibodies and Constructs

Affinity-purified antibodies against human cyclin A1, cyclin B1, Cdc2, hCDC20, and Plk1 were purchased from Santa Cruz. The anti-hCDH1 polyclonal rabbit serum was raised against two peptides derived from the hCDH1 protein: residues 65–79 and residues 108–123. The antisera were then affinity-purified against each peptide and used separately in immunoblotting (Harlow and Lane, 1988). Antibodies against both hCDH1 peptides recognized a single 55 kDa band in HeLa cell extracts. The rabbit anti-APC2 antibodies were raised against a C-terminal fragment (residues 701–823) of

human APC2. The rabbit anti-APC7 antibodies were made against a C-terminal fragment (residues 241–565) of human APC7.

Preparation of Synchronized HeLa Cell Extracts

For synchronization, HeLa S3 cells were grown in the presence of 2 mM thymidine (Sigma) for 18 hr, washed with PBS, and grown in fresh medium without thymidine for 8 hr. Thymidine was added again to 2 mM to block cells at G1/S. After another 18 hr, cells were transferred to fresh medium, and samples were harvested every 1–2 hr for a period of 28 hr. To arrest cells in mitosis, cells were first treated with 2 mM thymidine, released into fresh medium for 3–4 hr, and then blocked with medium containing 100 ng/ml nocodazole for 12 hr. Cells were washed with PBS, transferred into fresh medium for 1 hr, and harvested.

For the preparation of extracts, cells were lysed with four volumes of the NP-40 lysis buffer (50 mM Tris-HCl [pH 7.7], 150 mM NaCl, 0.5% NP-40, 1 mM DTT, 10% glycerol, 0.5 μM okadaic acid, and 10 μg/ml each of leupeptin, pepstatin, and chymostatin. The lysate was then centrifuged for 1 hr at 100,000 g to make the high–speed supernatant (S100).

Fractionation of Mitotic *Xenopus* Egg

Mitotic *Xenopus* egg extracts and the high–speed supernatants (S100) were prepared as previously described (King et al., 1995). Diluted S100 supernatant was applied to a 50 ml Resource Q column (Pharmacia) equilibrated with buffer Q-A (20 mM Tris-HCl [pH 7.7], 100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM DTT) and eluted with buffer Q-B (Q-A + 1 M KCl) with a 0%–60% gradient in a volume of 300 ml. Each column fraction was desalted with PD10 columns (Pharmacia), concentrated in Centriprep-10 concentrators (Amicon), and tested for their abilities to activate the interphase *Xenopus* APC.

APC Activation and Ubiquitination Assays

To purify interphase APC, the anti-CDC27 antibody beads were incubated with 10 volumes of interphase *Xenopus* egg extract for 2 hr at 4°C and washed five times with Q-A containing 500 mM KCl and 0.5% NP-40 and twice with Q-A. The interphase APC beads were then incubated with either mitotic column fractions or in vitro translated hCDC20 or hCDH1 proteins for 1 hr at room temperature, washed twice with Q-A, and assayed for cyclin ubiquitination activities. To purify hCDC20 and hCDH1 proteins, both proteins were tagged with two copies of HA epitope at their amino termini and in vitro translated in reticulocyte lysates (Promega). The lysates (400 μl each) were then incubated with 20 μl of protein A beads containing 15 μg of anti-HA antibody (Santa Cruz) for 2 hr at room temperature, washed seven times with Q-A containing 500 mM KCl and 0.5% NP-40 and three times with Q-A, and eluted with 250 μl of 0.2 mM HA peptide (Santa Cruz) overnight at 4°C. The eluates (0.5 μl each) were analyzed by silver-stained SDS-PAGE. The eluates were concentrated by 6-fold in microconcentrators (Amicon) and used in activation assays.

The substrates used in the assays were an N-terminal fragment of *Xenopus* cyclin B (residues 1–102) and the 1–102 fragment with the D-box (residues 36–44) deleted. Ubiquitination reaction mixture contains an energy regenerating system, 1.25 mg/ml of bovine ubiquitin (Sigma), 12.5 ng/ml of radiolabeled substrate, 200 μg/ml wheat E1, 50 μg/ml UBCx, and 2 μl of APC beads. The reactions were incubated at room temperature for 1 hr and analyzed by SDS-PAGE (5%–15% gradient gels) followed by phosphorimaging. For the kinetic experiments, samples were taken at indicated timepoints and quenched with SDS buffer.

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