APC2 Cullin Protein and APC11 RING Protein Comprise the Minimal Ubiquitin Ligase Module of the Anaphase-promoting Complex

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In mitosis, the anaphase-promoting complex (APC) regulates the onset of sister-chromatid separation and exit from mitosis by mediating the ubiquitination and degradation of the securin protein and mitotic cyclins. With the use of a baculoviral expression system, we have reconstituted the ubiquitin ligase activity of human APC. In combination with Ubc4 or UbcH10, a heterodimeric complex of APC2 and APC11 is sufficient to catalyze the ubiquitination of human securin and cyclin B1. However, the minimal APC2/11 ubiquitin ligase module does not possess substrate specificity, because it also ubiquitinates the destruction box deletion mutants of securin and cyclin B1. Both APC11 and UbcH10 bind to the C-terminal cullin homology domain of APC2, whereas Ubc4 interacts with APC11 directly. Zn²⁺-binding and mutagenesis experiments indicate that APC11 binds Zn²⁺ at a 1:3 M ratio. Unlike the two Zn²⁺ ions of the canonical RING-finger motif, the third Zn²⁺ ion of APC11 is not essential for its ligase activity. Surprisingly, with Ubc4 as the E2 enzyme, Zn²⁺ ions alone are sufficient to catalyze the ubiquitination of cyclin B1. Therefore, the Zn²⁺ ions of the RING finger family of ubiquitin ligases may be directly involved in catalysis.

INTRODUCTION

The orderly progression through mitosis relies on the sequential degradation of several cell cycle regulatory proteins mediated by the mitotic ubiquitination system (King et al., 1996a; Zachariae and Nasmyth, 1999; Nasmyth et al., 2000). This system first initiates the onset of sister-chromatid separation by ubiquitinating the anaphase inhibitor or securin (Nasmyth et al., 2000). Degradation of the ubiquitinated securin in turn activates the separase, which then cleaves a subunit of the cohesin complex, resulting in the loss of sister-chromatid cohesion and the onset of anaphase (Uhlmann et al., 1999, 2000; Waizenegger et al., 2000). The same machinery also mediates the ubiquitination and destruction of mitotic cyclins, leading to the inactivation of the cdc2 activity and the exit from mitosis (King et al., 1996a; Morgan, 1999; Zachariae and Nasmyth, 1999).

In this system, a large protein complex, called the anaphase-promoting complex (APC) or cyclosome, functions as the ubiquitin ligase (E3) (Irniger et al., 1995; King et al., 1995; Sudakin et al., 1995; Tugendreich et al., 1995). In the presence of the ubiquitin-activating enzyme (E1) and certain ubiquitin-conjugating enzymes (E2s) such as Ubc4 and UbcH10 (called UbcX in Xenopus and E2-C in clams), APC catalyzes the attachment of ubiquitin to the lysine side chains of securin and mitotic cyclins (King et al., 1995; Aristarkhov et al., 1996; Yu et al., 1996). The polyubiquitinated APC substrates are then targeted to the 26S proteasome for degradation (Hershko and Ciechanover, 1998).

To ensure that APC substrates are degraded with proper timing, the ubiquitin ligase activity of APC is tightly regulated during the cell cycle (King et al., 1996a; Morgan, 1999). Significant progress has been made toward the understanding of the regulation of APC during the cell cycle. APC is turned on during mitosis, remains active through most of G1, and is rapidly inactivated at the G1/S transition (Fang et al., 1998; Kramer et al., 1998). The activity profile and substrate specificity of APC can be partially explained by the transient association of two related regulatory factors, Cdc20 and Cdh1, at specific stages of the cell cycle (Sigrist and Lehner, 1997; Schwab et al., 1997; Visintin et al., 1997; Fang et al., 1998; Kramer et al., 1998). Cdc20 binds to APC in mitosis, whereas Cdh1 interacts with APC both in mitosis and the G1 phase (Fang et al., 1998; Kramer et al., 1998). Binding of either Cdc20 or Cdh1 to APC increases the activity of APC drastically (Fang et al., 1998; Kramer et al., 1998). Although the exact mechanism by which Cdc20 and Cdh1 contribute to
the ubiquitination reactions is unknown, several recent ob-
servations suggest that they might be directly involved in
substrate recruitment (Schwab et al., 1997; Sigrist and Leh-
ner, 1997; Visintin et al., 1997; Burton et al., 2001; Hillioli et al.,
2001; Pfleger et al., 2001).
In contrast to the cell cycle regulation of APC, little is
known about the mechanism by which APC catalyzes the
ubiquitination reaction. This is partially due to the fact that
the vertebrate APC contains at least 11 subunits (Yu et al.,
1998; Grossberger et al., 1999; Gmachl et al., 2000). No clues
about the biochemical functions of most APC subunits can
be inferred from examining their amino acid sequences.
However, sequence analysis of APC2 and APC11 has re-
vealed homology with proteins involved in other ubiquiti-
nation systems, particularly the Skp1-Cullin-F-box (SCF)
pathway (Yu et al., 1998; Zachariae et al., 1998; Deshaies,
1999; Ohta et al., 1999; Skowrya et al., 1999; Gmachl et al.,
2000).
APC2 contains a region that is similar to a sequence in
cullins, and thus is a distant member of the cullin family (Yu
et al., 1998; Zachariae et al., 1998). The cullin family of pro-
teins is essential for the ubiquitination of G1 cyclins, cyclin-
dependent kinase inhibitors, and other important regulatory
proteins in yeast and mammals (Kipreos et al., 1996; Willems
et al., 1996; Feldman et al., 1997; Lypina et al., 1998; Latres
et al., 1999; Tan et al., 1999). A cullin protein in budding yeast,
Cdc53p, is a part of the SCF ubiquitin ligase complex, which
targets phosphorylated Sic1p and G1 cyclins for degradation
in late G1 (Willems et al., 1996; Feldman et al., 1997). Similar
SCF complexes containing cullin proteins have been found
in mammals; they mediate the degradation of a variety of
substrates, including IkB and E2F (Marti et al., 1999; Tan
et al., 1999). APC11 contains a Zn2+ binding motif referred to
as the RING-H2 finger and is homologous to Rbx1/Roc1/
Htr1 that physically interacts with Cull1 in mammals and
Cdc53p in yeast (Zachariae et al., 1998; Kamura et al., 1999;
Ohta et al., 1999; Seol et al., 1999; Skowrya et al., 1999; Gmachl
et al., 2000). Because the Cull1/Rbx1 heterodimeric complex
is the functional core of the SCF ubiquitin ligases, APC2 and
APC11, by analogy, might also be directly involved in ca-
talyis (Seol et al., 1999). In fact, it has recently been shown
that APC11 alone is sufficient to ubiquitinate APC substrates
in the presence of Ubc4 (Gmachl et al., 2000; Leverson et al.,
2000). Surprisingly, UbcH10 does not support the ubiquiti-
nation reaction mediated by APC11 (Gmachl et al., 2000; Leverson
et al., 2000).
To gain insights into the catalytic mechanism of APC, we
have reconstituted the ubiquitin ligase activity of APC. The
minimal ligase module of APC is the heterodimeric complex
of the cullin-related protein APC2 and the RING finger
protein APC11. Therefore, APC is a member of the expand-
ing family of cullin-RING finger-based ubiquitin ligases.
Although APC11 alone can ubiquitinate APC substrates
with the use of Ubc4 as the E2 enzyme, the APC2/11 com-
plex is required to catalyze the ubiquitination of APC sub-
strates in the presence of UbcH10. Ubc4 interacts directly
with APC11, whereas UbcH10 does not. Instead, UbcH10
binds to the C-terminal cullin domain of APC2. We further
demonstrate that APC11 contains a third Zn2+-binding site,
in addition to the two Zn2+ ions of the canonical RING-H2
finger. Interestingly, Zn2+ ions alone can catalyze the ubi-
quitination of APC substrates with the use of Ubc4 as the E2
enzyme, suggesting that Zn2+ ions of the RING finger-
containing ubiquitin ligases might be directly involved in
catalysis.

MATERIALS AND METHODS

Protein Expression with Use of Baculoviral System
In addition to the eight human APC subunits (APC1–8) reported
previously (Yu et al., 1998), two more subunits were cloned with the
use of polymerase chain reaction (PCR), APC10 and APC11, based
on their sequence similarity to the yeast APC subunits. The recom-
binant baculoviruses were constructed with the Bac-to-Bac system
(Invitrogen, Carlsbad, CA). Briefly, the human genes encoding APC
subunits (APC1–11), Cdc20, and Cdh1 were amplified by PCR and
cloned into the pFastBac-pFastBac-HI, or pFastBac-GST vectors
with the use of suitable restriction sites for the production of un-
tagged, N-terminal His-tagged, or N-terminal GST-tagged pro-
teins. These plasmids were then transformed into the Escherichia coli
strain DH10Bac and the desired bacmids were isolated. These bac-
mids were transfected into adherent Sf9 cells for the packaging of
baculoviruses. To limit the number of viruses used in the coinfe-
ction assays, two recombinant baculoviruses each harboring two APC
genes (APC1/8, APC2/7, APC3/6, and APC4/5) were also made with the use of the
pFastBacDual vector that contains two independent promoters to
transcribe both genes simultaneously. The initial viral stocks were
then amplified with two successive rounds of infection of Sf9 cells in
suspension. The titers of the final viral stocks were typically 1–5 ×
10^9/ml, as measured with the Rapid Titer kit (CLONTECH, Palo
Alto, CA). Small samples were taken during the second round of
amplification and checked for protein production and solubility by
immunoblotting with the relevant antibodies. The production of all
10 APC subunits, Cdc20, and Cdh1 proteins in soluble form was
verified by either Coomassie staining or immunoblotting.

For the coinfection experiments, Sf9 or Hi5 insect cells were
infected with a Multiplicity of infection of 5–10 for each virus for
40–50 h. Cells were harvested and lysed by Dounce homogeniza-
tion. The lysates were cleared by centrifugation at 30,000 × g for 1 h.
The human APC proteins were then purified via the appropriate tags
and assayed for ubiquitin ligase activity or binding to other pro-
teins. When necessary, the APC proteins were further purified by
anion exchange and/or gel filtration chromatography.

Ubiquitination Assay

For the ubiquitination assays involving the intact APC, the α-APC3
(Cdc27) beads were incubated with 10 volumes of insect cell Xeno-
pus egg extracts for 2 h at 4°C and washed five times with XB (10
mM HEPES, pH 7.7, 100 mM KCl, 0.1 mM CaCl2, 1 mM MgCl2, 50
mM sucrose) containing 500 mM KCl and 0.5% NP-40 and twice
with XB. The interphase APC beads were then incubated for 1 h at
room temperature with human Cdc20 or Cdh1 proteins. After in-
cubation, the APC beads were again washed twice with XB and
assayed for ubiquitin ligase activity. For the assays with the recon-
mstituted APC, 5 μl of Ni2+-nitrilotriacetic acid (NTA) beads were
incubated with 500 μl of the insect cell lysate containing the appro-
priate Hisα-tagged APC proteins, washed five times with XB, and
assayed for ubiquitin ligase activity.

Each ubiquitination assay was performed in a volume of 5–10 μl.
The reaction mixture contained ATP, 150 μM of bovine ubiquitin, 5
μM of the Myc-tagged human securin, or an N-terminal fragment
of human cyclin B1, 5 μM of human E1, 2 μM of Ubc4 or UbcH10, and
2–5 μl of the APC beads. The reactions were incubated at room
temperature for 1 h, quenched with SDS sample buffer, and anal-
alyzed by SDS-PAGE followed by immunoblotting with α-Myc.

Cyclin Degradation Assay

The Xenopus egg extracts were prepared as described previously
(Murray, 1991). To assay cyclin degradation, the N-terminal frag-
ment of human cyclin B1 (residues 1–102) with a Myc-tag and ubiquitin were added to the mitotic extracts at final concentrations of 100 nM and 150 μM, respectively. Aliquots of the reaction mixture were quenched by SDS sample buffer at the indicated time, separated by SDS-PAGE, and blotted with α-Myc.

**Protein Binding Assay**

To assay the binding among APC2, APC11, Ubc4, and UbcH10, one of the binding partners was expressed either in bacteria or insect cells as His6-tagged proteins. The other partner was in vitro translated in reticulocyte lysate in the presence of [35S]methionine. Purified His6-tagged proteins were bound to Ni2+-NTA beads, incubated with the 35S-labeled proteins, and washed three times with Tris-buffered saline containing 0.05% Tween. The 35S-labeled proteins retained on beads were analyzed by SDS-PAGE followed by autoradiography. The vectors encoding the APC2 fragments were constructed by PCR. The APC11 mutants were made with the use of the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

**Zn**

The purified APC2e/11 protein was dialyzed against TNG buffer (10 mM Tris-HCl, pH 8.0, 200 mM NaCl, and 5% glycerol). The concentration of APC2e/11 was determined with the use of UV-VIS spectroscopy and Bradford assays (Bio-Rad, Hercules, CA). The Zn2+ ions bound by APC2e/11 were released by p-hydroxymercuri-phenylsulfonic acid. The released Zn2+ was then coordinated by 4-2-pyridylazoresorcinol (PAR), and the resulting Zn2+-PAR complex absorbed light at 500 nm with an extinction coefficient of 6.6 × 10^4 M^-1 cm^-1. Specifically, aliquots of 1 mM p-hydroxymercuri-phenylsulfonic acid were successively added to a mixture containing 1.5 μM of APC2e/11 and 100 μM of PAR, until a plateau of OD500 was reached. The maximum value of OD500 divided by the extinction coefficient then yielded the concentration of Zn2+.

**RESULTS**

**Reconstitution of Ubiquitin Ligase Activity of Human APC**

To investigate the mechanism of APC, we coinfected Hi5 insect cells with multiple recombinant baculoviruses harboring 10 APC genes and the cofactors Cdc20 or Cdh1. Because both APC2 and APC11 were required for the reconstituted APC activity, we tested whether they interacted with each other in the absence of the rest of the APC subunits. To characterize the immediate binding partners of APC11 in the APC complex, the GST-APC11 virus was coinfectected with each of the other viruses in a pairwise manner. GST-APC11 and its associated subunits were then purified with glutathione-Sepharose beads and analyzed by SDS-PAGE followed by Coomassie staining and immunoblotting. As shown in Figure 2A, APC11 binds tightly to APC2 and weakly to APC6. The identities of APC2 and APC6 were verified by immunoblotting (our unpublished data).

Because Cdc20 and Cdh1 are positive regulators of APC, it was surprising to us that Cdc20 or Cdh1 were not required for the ubiquitin ligase activity of the reconstituted APC. We therefore compared the reconstituted APC activity with that of the intact APC from Xenopus egg extracts. Nearly all APC substrates contain the destruction box (D-box) or the KEN-box motifs, which are required for the correct ubiquitination and degradation of these substrates (King et al., 1996b; Pfleger and Kirschnner, 2000). Cdc20 and Cdh1 have been shown to confer the D-box and KEN-box specificity of APC (Burton et al., 2001; Hilioti et al., 2001; Pfleger et al., 2001). The activities of the intact APC-Cdc20 and APC-Cdh1 from Xenopus were assayed with cyclin B1 as the substrate. As shown in Figure 1B, purified Cdc20 and Cdh1 proteins greatly stimulated the ligase activity of the intact interphase Xenopus APC with UbcH10 as the E2 enzyme and wild-type cyclin B1 as substrate. However, neither the intact APC-Cdc20 nor APC-Cdh1 significantly ubiquitinated a D-box deletion mutant of cyclin B1 (ΔDB-cyclin B1). Similar results were obtained with Ubc4 as the E2 enzyme, although the patterns of cyclin B-ubiquitin conjugates formed by the two enzymes were different. Ubc4 appeared to be more processive than UbcH10 in the presence of either intact APC-Cdc20 or APC-Cdh1.

Because Cdc20 and Cdh1 were not essential for the reconstituted APC activity, we tested whether the ligase activity obtained with overexpressed APC proteins conferred D-box specificity, similar to the intact APC. Not surprisingly, the reconstituted human APC ubiquitinated ΔDB-cyclin B1 equally efficiently, indicating that the reconstituted activity did not possess substrate specificity (Figure 1C). This activity also required the presence of APC2 and APC11. Therefore, we reconstituted the minimal ligase activity of APC, which lacked D-box dependency. At present, we do not know the exact cause for the lack of D-box dependency of our reconstituted APC. However, several factors might contribute to this. First, the reconstituted APC might not have the correct quaternary arrangement of all the relevant subunits. Second, the set of subunits used to reconstitute the APC is not complete. Additional human APC subunits are required for the proper function of the reconstituted APC. Finally, it is also possible that the high concentrations of the reconstituted APC and the substrates in our in vitro reactions may have eliminated the need for high-affinity interactions between APC and substrates. We are currently investigating these possibilities.
unpublished data). We therefore used a His$_6$-tagged APC10 virus to infect Sf9 cells together with the GST-APC11 virus. The APC10 protein was then purified with Ni$^{2+}$-NTA beads and analyzed by SDS-PAGE. APC10 binds tightly to GST-APC11 as revealed by Coomassie staining (Figure 2B) and immunoblotting with $\alpha$-GST antibody (Figure 2C). Several large proteins contain the so-called DOC domains that are similar in sequence to APC10; some of these proteins also contain cullin homology domains or homologous to E6-AP C terminus (HECT) domains (Grossberger et al., 1999). E6-AP, the founding member of a family of proteins containing HECT domains, mediates the HPV E6-dependent degradation of p53 (Scheffner et al., 1995). It is likely that the DOC domains of these large multidomain proteins might also be involved in binding to yet unidentified RING-finger proteins.

**Figure 1.** Reconstitution of the ubiquitin ligase activity of APC. (A) Hi5 insect cells were coinfected with baculoviruses encoding the indicated APC subunits. The expressed His$_6$-tagged APC proteins were isolated from the insect cell lysate with the use of the Ni$^{2+}$-NTA beads and assayed for ubiquitin ligase activity in the presence of UbcH10. The reaction mixture was separated on SDS-PAGE and blotted with $\alpha$-Myc to detect the C-terminally Myc-tagged cyclin B1 protein. Omission of the APC2 (lane 3) or APC11 (lane 11) viruses from the coinfection resulted in the loss of ubiquitin ligase activity. (B) Ubiquitin ligase activity of the intact APC. Either UbcH10 (lanes 1–6) or Ubc4 (lanes 7–12) was used as the E2 enzyme. To determine the D-box dependency of APCCdc20 and APCCdh1, either the wild-type (WT) cyclin B1 (lanes 1–3 and 7–9) or the D-box deletion mutant ($\Delta$DB) of cyclin B1 (lanes 4–6 and 10–12) was used as substrates. (C) Same as A except that $\Delta$DB-cyclin B1 was used as the substrate instead of the wild-type cyclin B1 protein.
To identify subunits that interact with APC2, the His6-tagged APC2 virus was used to infect Sf9 cells together with other viruses. The APC2 protein was then purified with Ni2+/H11001-NTA beads and analyzed by SDS-PAGE. We confirmed that APC2 interacts with APC11, and found no other strong interactions between APC2 and the rest of APC subunits (our unpublished data). Therefore, APC2 and APC11 formed a complex in the absence of the other APC subunits.

We next tested whether the subcomplex of APC2 and APC11 (APC2/11) was sufficient to support ubiquitination of APC substrates. With the use of UbcH10 as E2, APC2/11 catalyzed the ubiquitination of human securin, whereas either APC2 or APC11 alone had no activity (Figure 3A). Consistent with previous reports, APC11 alone expressed either in bacteria or insect cells was sufficient to ubiquitinate human securin in the presence of Ubc4 (Figure 3B). Similar data were obtained with the use of human cyclin B1 as the substrate (our unpublished data). Therefore, APC2/11 represents the minimal ligase module of APC, because it supports the ubiquitination of APC substrates with the use of either Ubc4 or UbcH10 as E2s.

Ubc4 Interacts with APC11, whereas Ubhc10 Binds to APC2

Both Ubc4 and UbcH10 support the ubiquitination reactions catalyzed by APC in an additive manner (Yu et al., 1996). It is unclear which enzyme is the physiological E2 of the APC pathway. Microinjection of a UbcH10 dominant-negative mutant protein into mammalian cells arrested cells in mitosis (Townsley et al., 1997). In addition, mutation of the Schizosaccharomyces pombe homolog of UbcH10, UbcP4, caused accumulation of cells in mitosis, similar to mutations of APC subunits (Osaka et al., 1997). These findings suggest that UbcH10 might be involved in the mitotic degradation system in living cells. However, in budding yeast, mutations of either the Ubc4/5 family E2s or the UbcH10 homolog Ubc11 did not cause obvious mitotic phenotype (Townsley et al., 1997).

Figure 2. APC11 interacts with APC2 and APC10. (A) GST-APC11 baculovirus was coinfected with APC1/8 (a single baculovirus encoding both APC1 and APC8), APC2/7, APC3/6, APC4/5, or Cdh1 viruses in a pairwise manner into Sf9 cells. GST-APC11 and its interacting proteins were purified on glutathione beads, separated on SDS-PAGE, and stained with Coomassie Blue. GST was added to the lysates of APC1/8, APC2/7, APC3/6, APC4/5, and Cdh1 and used as controls. APC11 interacted strongly with APC2 (lane 3) and weakly with APC6 (lane 5). As determined by mass spectrometry, the band at 55 kDa belonged to α/β-tubulin, which presumably associated nonspecifically with GST-APC11. (B) His6-tagged APC10 baculovirus was coinfected with the GST-APC11 virus into Sf9 cells. His6-APC10 and its interacting proteins were purified on Ni2+/H11001-NTA beads, separated on SDS-PAGE, and stained with Coomassie Blue. As controls, Ni2+/H11001-beads were added to lysates infected with GST-APC11 alone. The α/β-tubulin proteins again copurified with APC10 and APC11. (C) To verify the interaction between APC10 and APC11, the same samples from B were blotted with α-GST, confirming the identity of the GST-APC11 band.

Figure 3. Heterodimeric complex of APC2 and APC11 possesses ubiquitin ligase activity. (A) Hi5 cells were either infected with His6-APC2 (lanes 2 and 7) and His6-APC11 (lanes 3 and 8) viruses individually, or coinfected with the His6-APC2 and His6-APC11 viruses (lanes 4 and 9). The APC2 and APC11 proteins were purified with Ni2+/H11001-NTA beads and assayed for ubiquitination activity with the use of UbcH10 as the E2 enzyme and wild-type human securin (lanes 1–5) or a D-box deletion mutant (ΔDB) of securin (lanes 6–10) as the substrates. Bacterial expressed GST-APC11 protein purified with glutathione-agarose beads was also tested for ligase activity (lanes 5 and 9). (B) Same as A except that Ubc4 was used as the E2 enzyme instead of UbcH10.
bind to APC11. In contrast, Ubc4 associated directly with APC11. It did not exhibit significant binding toward APC2. This finding explains why APC2 is only required for UbcH10-catalyzed reactions. The two E2s may recognize different binding determinants within the APC2/11 ligase module. The fact that UbcH10 did not bind APC11 is consistent with previous structural studies on the interactions between the Cbl RING domain and UbcH7, an E2 of the Ubc4 subfamily (Zheng et al., 2000). The two loops that are critical for binding to RING domains are conserved between Ubc4 and UbcH7 (Figure 4C). On the other hand, UbcH10 contains quite divergent amino acid sequences in these two loops. Therefore, Ubc4 and UbcH10 may be recruited to the intact APC with distinct mechanisms: Ubc4 recognizes APC11 initially, whereas UbcH10 first interacts with APC2. However, it remains possible that, once they are bound to APC, Ubc4 and UbcH10 occupy a similar site on APC and use a similar mechanism for transferring ubiquitin.

**Cullin Domain of APC2 Interacts with APC11 and UbcH10**

We next mapped the regions within APC2 that interact with APC11 and UbcH10. A series of truncation mutants of APC2 were constructed and tested for binding to APC11 and UbcH10. A C-terminal fragment of APC2, APC2e, spanning residues 549–822, was sufficient for binding to APC11 and UbcH10 (Figure 5A). This region almost coincides with the cullin homology region of APC2 that includes residues 512–750 (Yu et al., 1998). Interestingly, even although APC2 and APC11 formed an active complex when they were coexpressed in insect cells, the full-length APC2 protein did not bind to APC11 in this assay. This is consistent with the fact that, when APC2 and APC11 were expressed individually in insect cells and mixed after purification, no ubiquitin ligase activity was observed. Therefore, the full-length APC2 protein could not form a complex with APC11 post-translationally. However, smaller fragments of APC2 were able to bind to APC11 (Figure 5A).

Because APC2e binds to both APC11 and UbcH10, we examined whether the APC2e/11 complex was an active ubiquitin ligase. The APC2 fragments were coexpressed with APC11 in insect cells, and assayed for their ability to ubiquitinate cyclin B1 in the presence of UbcH10. The APC2b and APC2e fragments ubiquitinated cyclin B1 efficiently (Figure 5B); both of these fragments retained the ability to bind to APC11 and UbcH10 (Figure 5A). Therefore, a complex of the cullin domain of APC2 and the RING finger protein APC11 is sufficient to catalyze ubiquitination of APC substrates, albeit with decreased efficiency.

One potential caveat of reconstituting the APC activity in insect cells is that certain insect APC proteins might associate with the expressed human APC proteins and contribute to the observed ligase activity. To rule out this possibility, we purified the APC2e/11 complex to homogeneity (Figure 5C) and determined the native size of the APC2e/11 complex by gel filtration chromatography and dynamic light scattering experiments. APC2e/11 cofractionated as a single species with an apparent molecular mass of 50 kDa on the gel filtration column (our unpublished data). Based on the intensity of staining on SDS-PAGE (Figure 5C), we estimated that APC2e and APC11 formed a complex of 1:1 stoichiometry. The calculated molecular mass of the comple-
plex is thus 50 kDa. Based on the light scattering experiment, the APC2e/11 complex was mono-dispersed with an apparent molecular mass of 46 kDa. Therefore, it is extremely unlikely that the APC2e/11 complex contains any insect proteins.

**Zn\(^{2+}\)-binding of APC11 Is Essential for Its Ubiquitin Ligase Activity**

Because other RING finger proteins are known to coordinate Zn\(^{2+}\) ions, APC11 may also bind Zn\(^{2+}\). However, this has not been demonstrated experimentally. We thus performed a Zn\(^{2+}\)-binding assay on the purified APC2e/11 complex (Yu and Schreiber, 1995). Surprisingly, based on four measurements, we found that APC2e/11 bound Zn\(^{2+}\) at a molar ratio of 3.2 ± 0.2. Similar results were obtained with purified GST-APC11 protein expressed in bacteria. Therefore, it appeared that, in addition to the two Zn\(^{2+}\) ions coordinated by the canonical RING finger motif, APC11 contained a third Zn\(^{2+}\)-binding site (Figure 6A). Sequence alignment of the APC11 and Rbx1 proteins from various organisms reveals that three cysteines (Cys 34, Cys 37, and Cys 44 in human APC11) and one histidine (His 58 in human APC11) are conserved among these proteins (Figure 6A). These conserved residues do not belong to the canonical RING-H2 finger motif, and thus are good candidates for coordinating the third Zn\(^{2+}\) ion.

To determine the residues that coordinate Zn\(^{2+}\) ions, all cysteines and histidines of human APC11 were individually mutated to serines and alanines, respectively. Mutations of the Zn\(^{2+}\)-binding ligands of the RING finger motif markedly reduced the expression levels of these proteins in bacteria (our unpublished data). Similar results were obtained when Cys 34, Cys 37, Cys 44, and His 58 were mutated. In contrast, mutations of Cys 7, Cys 33, Cys 54, His 65, and His 72 had no effect on the expression levels of the APC11 protein (our unpublished data). These findings are consistent with the notion that Cys 34, Cys 37, Cys 44, and His 58 coordinate the third Zn\(^{2+}\) ion. It is possible that mutations of the Zn\(^{2+}\)-binding ligands destabilized the tertiary structure of APC11, resulting in the reduced level of expression of APC11. Obviously, loss of expression of APC11 mutant proteins in bacteria can be caused by factors other than protein folding. Because the APC11 mutant proteins that involve the putative Zn\(^{2+}\)-binding ligands could not be obtained at sufficient purity and quantity, we could not determine whether mutations of these ligands actually caused the loss of Zn\(^{2+}\)-binding.
When coexpressed with APC2, the APC11 mutants that did not affect Zn$^{2+}$-binding still possessed ubiquitin ligase activity toward cyclin B1 in the presence of UbcH10 (our unpublished data). Because the APC11 mutants that perturbed Zn$^{2+}$-binding were not expressed well, we could not compare the ubiquitin ligase activities of these mutants with those of the wild type or mutants that did not affect Zn$^{2+}$-binding. To circumvent this problem, we obtained all APC11 mutant proteins with the use of the in vitro transcription and translation system in rabbit reticulocyte lysate. Many RING finger-based ubiquitin ligases also autoubiquitinate in the presence of the proper E2 enzyme. We therefore tested the autoubiquitination activity of the APC11 mutants in the presence of Ubc4. The wild-type APC11 protein and the APC11 mutants that did not affect Zn$^{2+}$-binding were ubiquitinated efficiently when Ubc4 was added, based on the appearance of APC11-ubiquitin conjugates and the percentage of APC11 conjugated to ubiquitin (Figure 6B). Mutations of the eight Zn$^{2+}$-binding ligands of the RING finger motif greatly reduced the autoubiquitination activity of APC11 (Figure 6B). In contrast, mutations of Cys 34, Cys 37, Cys 44, and His 58 that coordinated the third Zn$^{2+}$ ion only slightly reduced the autoubiquitination activity of APC11 (Figure 6B). Thus, unlike the two Zn$^{2+}$ ions of the RING-H2 finger motif, the third Zn$^{2+}$ of APC11 might not be involved in catalysis (Figure 6B). This Zn$^{2+}$ ion may only be important for maintaining the structural integrity of APC11. We also tested the binding of all APC11 mutants to APC2e. None of the mutations affected the binding of APC11 to APC2 (our unpublished data). Because the N-terminal region of APC11 and Rbx1 proteins is conserved and this region is not present in other RING-finger proteins, we speculate that the N-terminal 20 residues of APC11 and Rbx1 proteins are involved in binding to APC2 and Cul1, respectively.

Zn$^{2+}$ Ions Alone Stimulate Activity of Ubc4 to Ubiquitinate Cyclin B1

Recently, many RING finger proteins have been shown to possess ubiquitin ligase activities. Despite the relatively low sequence homology outside the RING finger motif, several RING finger proteins use the Ubc4/5 family of E2 enzymes...
in the ubiquitination reactions (Joazeiro et al., 1999; Lorick et al., 1999; Gmachl et al., 2000; Leverson et al., 2000). This suggested to us that the Zn$^{2+}$ ions, the most obvious common feature of these RING finger proteins, might be directly involved in catalysis. Strikingly, we found that Zn$^{2+}$ ions alone stimulated the ability of Ubc4 to ubiquitinate cyclin B1 (Figure 7A). Several other divalent cations, such as Cd$^{2+}$, Co$^{2+}$, and Ni$^{2+}$, also enhanced the activity of Ubc4, whereas Mn$^{2+}$, Mg$^{2+}$, Ca$^{2+}$, and Yb$^{3+}$ had no effects (Figure 7, A and B). None of these cations stimulated the activity of UbcH10, which did not bind to RING proteins directly (our unpublished data). These findings further support the notion that Zn$^{2+}$ may be directly responsible for the activity of the RING finger-containing ubiquitin ligases.

We next quantitatively compared the activities of the intact APC$^{Cdc20}$, the reconstituted APC, and the Zn$^{2+}$ ions alone. Various concentrations of APC$^{Cdc20}$ were used in the in vitro ubiquitination assay with the use of cyclin B1 as the substrate. The ligase activity of APC was measured by the intensities of the cyclin-ubiquitin conjugates, which were normalized by the number of ubiquitin molecules in the conjugates (Figure 7C). The activity of the reconstituted APC at 5 nM was similar to that of the intact APC$^{Cdc20}$ at 90 nM, indicating that the intact APC$^{Cdc20}$ was 55 times more active than the reconstituted APC. Zinc ions at 100 μM exhibited ligase activity comparable to 25 nM of APC$^{Cdc20}$. Thus, the activity of zinc ions alone was 4000 weaker than that of the intact APC.

**DISCUSSION**

**APC as a Member of Cullin-RING Finger Family of Ubiquitin Ligases**

Among the three classes of enzymes of the ubiquitin pathway, the ubiquitin ligases are the most divergent, in terms of both composition and function (Hershko and Ciechanover, 1998). All ubiquitination systems use a single ubiquitin-activating enzyme (E1) for the first step of the reaction (Hershko and Ciechanover, 1998). Although certain ubiquitin-conjugating enzymes (E2s) are used in different ubiquitination systems, all E2 enzymes are homologous in sequence and contain the ubiquitin-conjugation (UBC) domain (Hershko and Ciechanover, 1998). In contrast, the ubiquitin ligases (E3s) are loosely defined as entities that collaborate with E1 and E2 to ligate ubiquitin chains on substrates. They can be unrelated in amino acid sequence and use distinct biochemical mechanisms for catalysis.

The E3s identified so far can be divided into three major families. The first family consists of HECT domain-containing proteins (Scheffner et al., 1995). The second family of E3s includes Ubr1, c-Cbl, Mdm2, IAPs, and other RING finger-containing proteins, which appear to contain both the E2-binding domain and the substrate recognition motif in a single polypeptide chain (Joazeiro et al., 1999; Xie and Varshavsky, 1999; Fang et al., 2000; Yang et al., 2000). In contrast, the third group of E3s consists of large protein complexes that contain a minimal ligase core of a cullin and RING-H2 heterodimer, such as the SCF and the VBC complexes (Deshayes, 1999; Kamura et al., 1999). Based on the data presented herein, the minimal ligase module of APC is comprised of APC2 (a distant member of the cullin family) and

![Figure 7.](image-url) Zn$^{2+}$ ions alone stimulate the ubiquitination activity of Ubc4. (A) Various concentrations of Zn$^{2+}$, Cd$^{2+}$, and other divalent cations were added to a reaction mixture containing E1, ubiquitin, Ubc4, ATP, and cyclin B1. Ubiquitination of cyclin B1 was analyzed by immunoblotting with α-Myc. (B) Various concentrations of Zn$^{2+}$ and Yb$^{3+}$ were added to a reaction mixture containing E1, ubiquitin, Ubc4, ATP, and cyclin B1. Ubiquitination of cyclin B1 was analyzed by immunoblotting with α-Myc. (C) Comparison of the ubiquitin ligase activities of the intact APC$^{Cdc20}$, the reconstituted APC, and zinc ions alone. The ligase activity of APC$^{Cdc20}$ was plotted against the concentration used in the assay. The activities of the reconstituted APC at 5 μM and zinc ions alone at 100 μM were indicated by closed circle and triangle, respectively.
APC11 (a RING-H2 finger protein). Therefore, APC belongs to the third group of ubiquitin ligases (Figure 8A).

**Substrate Recognition by APC and Its Regulation during Cell Cycle**

In addition to the fact that APC, SCF, and VBC ligase complexes all contain a cullin-RING finger heterodimeric ligase core, there is another analogy between APC and the other two systems (Figure 8A). The minimal ligase modules of these complexes are connected to various adaptor proteins that serve to recruit substrates. In the case of SCF complex, the substrate-binding proteins, such as Cdc4, Grr1, Skp2, and β-TRCP, contain the F-box motif and interact with Skp1, which in turn associates with the cullin protein Cdc53 or

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**Figure 8.** (A) APC belongs to the cullin-RING family of ubiquitin ligases. (B) Proposed role of Zn$^{2+}$ in Ubc4-catalyzed ubiquitination reactions. See DISCUSSION for details.
cycle, in essence, serves to inhibit the substrate binding or Cdh1 from APC during the S, G2, and prophase of the cell tightly regulated during the cell cycle. Dissociation of Cdc20 instead, it is the association of Cdc20 or Cdh1 to APC that is substrates are not required for their ef-
ficient ubiquitination. Both SCF and VBC complexes recognize post-translationally modified substrates: the F-box proteins of SCF complexes bind phosphorylated protein substrates, whereas the VHL protein of the VBC complex recognizes a novel hydroxyproline moiety of the HIF substrate (Skowrya et al., 1997; Ivan et al., 2001; Jaakkola et al., 2001). Therefore, the critical regulation of SCF and VBC pathways is at the level of the substrates. In contrast, although all APC substrates contain specific sequence motifs, such as the D-box and KEN-box, post-translational modifications of the APC substrates are not required for their efficient ubiquitination. Instead, it is the association of Cdc20 or Cdh1 to APC that is tightly regulated during the cell cycle. Dissociation of Cdc20 or Cdh1 from APC during the S, G2, and prophase of the cell cycle, in essence, serves to inhibit the substrate binding ability and thus the activity of APC (Fang et al., 1998).

Mechanism of Ubiquitin Transfer of APC-catalyzed Reactions
E3-catalyzed ubiquitination reactions may use two distinct mechanisms for conjugating ubiquitin to substrates. In the case of E6-AP-mediated ubiquitination of p53, Ubc4 (the E2 in the system) first transfers ubiquitin to E6-AP to form an E3-ubiquitin thioester, which then attaches the ubiquitin to the lysine residues of p53 (Scheffner et al., 1995). However, this does not seem to be the prevailing mechanism for all ubiquitination reactions. For the SCF complexes, it appears that the ubiquitination reactions do not involve the formation of an E3-ubiquitin thioester (Seol et al., 1999). Instead, the SCF complexes serve as a scaffold to bring together the E2 enzyme Cdc34 and the substrates. The RING protein Rbx1 of SCF enhances the ubiquitin transfer activity of Cdc34, and ubiquitin is then transferred directly from the Cdc34 thioester to substrates.

Aside from the analogy between APC and SCF, there is additional evidence to suggest that APC might also use the latter scaffolding mechanism for ubiquitin transfer. First, we and others were unable to detect a ubiquitin thioester of any APC subunits so far. Second, in the presence of active APC-Cdc20 or APC-Cdh1, Ubc4 seems to generate cyclin-ubiquitin conjugates of higher molecular weight than those generated by UbcH10 (Figure 1B) (Yu et al., 1996). The E3-thioester model would predict that, no matter which E2 is used, the same APC-ubiquitin thioester acts as the intermediate for relaying the ubiquitin to cyclin. The two E2s would then have little influence over the pattern of cyclin-ubiquitin conjugates. Therefore, the fact that Ubc4 and UbcH10 support the formation of cyclin-ubiquitin conjugates with different patterns favors the scaffolding model. One possible explanation for the higher molecular weight conjugates catalyzed by Ubc4 might be that Ubc4 transfers ubiquitin faster than UbcH10. Thus, during the residence time of substrate binding to APC, Ubc4 might recharge and transfer ubiquitin more frequently than UbcH10, thus forming higher molecular weight conjugates than UbcH10. Finally, all cysteines of APC11 have been mutated. Except the cysteines coordinating Zn2+ ions, mutation of other cysteines does not abolish the ubiquitin ligase activity of APC11 with Ubc4 as the E2. Therefore, it is unlikely that an APC-ubiquitin thioester is involved in these ubiquitination reactions.

Role of Zn2+ Ions in Catalysis
The Zn2+ ions of the RING finger E3 ligases are clearly essential for maintaining the structural integrity of these proteins. Besides the structural role, are the Zn2+ ions of the RING finger proteins also directly involved in catalysis, similar to many Zn2+-binding metalloenzymes? Two lines of evidence presented herein suggest that this might be the case. First, we found that APC11 binds a third Zn2+ ion, aside from the two Zn2+ ions that form the RING finger motif. Mutations of the residues that coordinate the third Zn2+ ion appear to destabilize the structure of APC11. Yet, these APC11 mutants still possess E3 ligase activity. In contrast, mutations of the Zn2+-binding residues of the canonical RING motif not only disrupt protein structure but also abrogate the ligase activity of APC11. This suggests that, in addition to the structural role, the two Zn2+ ions of the RING motif might be directly involved in catalysis. This notion is further strengthened by the unexpected finding that Zn2+ ions alone can stimulate the ability of Ubc4 to ligate ubiquitin to cyclin B1. Zn2+ might facilitate two steps in the ubiquitin ligation reaction (Figure 8B). It might stabilize the oxanion of the putative tetrahedral intermediate of the reaction. Along this line, it is worth noting that polycations can stabilize the autoubiquitination activity of Cdc34, and the polycations have been proposed to stabilize the oxanion intermediate (Seol et al., 1999; Zachariae and Nasmyth, 1999). Zn2+ might also promote the release of the active thiol group of the E2 from the tetrahedral intermediate. Consistent with this notion, several cations that can potentially coordinate oxanions, such as Cu2+ and Yb3+, do not have the same effect as Zn2+. Obviously, the mechanism by which Zn2+ ions at high concentrations catalyze the ubiquitination reaction may not mimic the mode of action of the RING-containing ubiquitin ligases. Further experiments are required to clarify these issues.

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3850 Molecular Biology of the Cell


