The Anaphase-Promoting Complex: Proteolysis in Mitosis and Beyond

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Summary

Key events in mitosis such as sister chromatid separation and subsequent inactivation of cyclin-dependent kinase 1 are regulated by ubiquitin-dependent proteolysis. These events are mediated by the anaphase-promoting complex (APC), a cell cycle-regulated ubiquitin ligase that assembles multubiiquitin chains on regulatory proteins such as securin and cyclins and thereby targets them for destruction by the 26S proteasome.

Introduction

Cyclin and the “Cell Cycle Clock”
While working at Woods Hole in the summer of 1982, Tim Hunt discovered a protein in rapidly dividing sea urchin embryos that was synthesized during interphase but suddenly destroyed during cell division. The cyclic expression pattern of this protein, therefore called cyclin (Evans et al., 1983), suggested immediately that proteolysis is key to its regulation. The observation that cyclin’s disappearance coincided with blastomere cleavage raised the interesting possibility that cyclin proteolysis might control some important aspect of cell division. This hypothesis seemed so attractive because a proteolytic mechanism could explain how a hypothetical mitotic activator could be irreversibly inactivated, thereby resetting the “cell cycle clock” after completion of cell division.

In the two decades that have passed since then, these ideas have turned into one of the central dogmas in our understanding of the eukaryotic cell division cycle. We refer to Hunt’s cyclin as cyclin B today and know that it and several related “mitotic cyclins” are central regulatory elements of an enzymatic switch that triggers entry into mitosis (Table 1). At the heart of this switch is cyclin B’s binding partner, the cyclin-dependent kinase 1 (CdK1). Cyclin B synthesis during interphase is essential for activation of CdK1 which, together with other mitotic kinases, initiates many of the early events of mitosis. The inactivation of CdK1 during anaphase and telophase is as important for exit from mitosis as CdK1 activation is for entry into mitosis. Without CdK1 inactivation chromosomes do not decondense, nuclear envelopes do not reassemble, and cells do not divide (reviewed by Peters et al., 1998). CdK1 inactivation is also essential for DNA replication in the subsequent cell cycle by allowing the formation of prereplicative complexes (Noton and Difffey, 2000), and partial CdK1 inactivation might be required for sister chromatid separation in anaphase in animal cells (Stemmann et al., 2001).

In presumably all eukaryotes, cyclin proteolysis is the major mechanism that is used to inactivate CdK1. It is now known that the enzymatic machinery that destroys cyclin is unusually complex, both with respect to its regulation and the number of components involved. This machinery does much more than just reset the cell cycle clock. It directly controls the chromosome by initiating sister chromatid separation in anaphase, and it regulates many other events inside and outside mitosis, possibly even in postmitotic differentiated cells. In this article, I discuss what we know today about the functions, regulation, and mechanisms of this pathway, with particular emphasis on the anaphase-promoting complex (APC) or cyclosome.

The Anaphase-Promoting Complex

Cyclin is destroyed by ubiquitin-dependent proteolysis, i.e., it is targeted for degradation by covalent attachment of a multubiiquitin chain, allowing its recognition by the 26S proteasome (Glotzer et al., 1991; Hershko et al., 1991). The 26S proteasome then degrades cyclin, leaving the catalytic CdK1 subunit intact. Cyclin ubiquitination reactions depend on at least four components (reviewed in Morgan, 1999; Peters, 1999; Zachariae and Nasmyth, 1999): the universal ubiquitin-activating enzyme E1, either one of two ubiquitin-conjugating enzymes (E2s), called UbcH5 and UbcH10 in human cells, the APC, and one of several APC activator proteins such as Cdc20 and Cdh1 (see Table 2 for names used in other species). The E1 enzyme transfers activated ubiquitin residues to UbcH5 or UbcH10, which then assemble ubiquitin chains on cyclin with the help of the APC. The APC was discovered as a mitosis and cyclin B specific ubiquitin ligase in clam and Xenopus extracts (King et al., 1995; Sudakin et al., 1995) and through the isolation of budding yeast mutants that are defective in the degradation of mitotic cyclins (Imliger et al., 1995). The clam enzyme was called cyclosome to illustrate the cyclic activation and inactivation of the cyclin degradation pathway (Sudakin et al., 1995), whereas the Xenopus and yeast ubiquitin ligase was called anaphase-promoting complex to illustrate its essential role in anaphase initiation (Imliger et al., 1995; King et al., 1995).

The APC is a high molecular mass complex composed of at least 11 subunits, but it is only fully active as a ubiquitin ligase once it has bound to Cdc20, Cdh1, or related activators, resulting in distinct assemblies called, for example, APC(Cdc20) or APC(Cdh1). Since Cdc20 and Cdh1 can bind to APC substrates, they may activate ubiquitination reactions by recruiting substrates to the APC (see below). APC-mediated ubiquitination depends on either one of two rather poorly defined sequence elements in the substrate, the destruction box (D box) and the KEN box (Glotzer et al., 1991; Pfeffer and Kirschner, 2000). D boxes were first identified in the N termini of mitotic cyclins and the KEN box in Cdc20, but both these elements are found either singly or in combination in all APC substrates known to date.

Not surprisingly, the ubiquitination of cyclin is regulated during the cell cycle, whereas proteasomal degradation of ubiquitinated cyclin does not appear to be.

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Table 1. Cyclin-Dependent Kinases in Budding Yeast and Animal Cells

<table>
<thead>
<tr>
<th>Cell Cycle Function</th>
<th>Budding Yeast</th>
<th>Animal Cells</th>
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<tbody>
<tr>
<td></td>
<td>CDK</td>
<td>APC Substrate</td>
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<tr>
<td>G1 phase</td>
<td>Cln1-Cdc28</td>
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</tr>
<tr>
<td></td>
<td>Cln2-Cdc28</td>
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<tr>
<td></td>
<td>Cln3-Cdc28</td>
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<tr>
<td>S phase</td>
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</tr>
<tr>
<td></td>
<td>Clb6-Cdc28</td>
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</tr>
<tr>
<td>Mitosis</td>
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<td></td>
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<tr>
<td></td>
<td>Clb3-Cdc28</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>Clb4-Cdc28</td>
<td>yes</td>
</tr>
</tbody>
</table>

*a Cyclin D1 may be degraded in an APC-dependent manner in response to DNA damage (Agami and Bernards, 2000).
*b Cyclin A has S phase-promoting function in vertebrate cells, but in Drosophila it promotes mainly entry into mitosis.

The regulated step in this ubiquitination pathway is the activation of the APC by its interaction with Cdc20 and Cdh1 (Figure 1). In mitosis, APC is activated by binding to Cdc20, and this is dependent on high Cdk1 activity. By allowing Cdk1 activation in prophase, cyclin therefore sows the seeds of its own destruction later in mitosis. Cyclin B degradation begins in metaphase and continues while sister chromatids separate and then move toward opposite spindle poles in anaphase (Clute and Pines, 1999). In most species and cell types, Cdc20 is also degraded during this period and replaced by Cdh1, which keeps the APC active until the end of the subsequent G1 phase. In early mitosis, phosphorylation of Cdh1 prevents its interaction with the APC. The binding of Cdh1 to the APC during mitotic exit therefore depends on Cdh1 dephosphorylation. At the G1-S transition, Cdh1 is rephosphorylated and thereby dissociated from the APC, allowing the reaccumulation of cyclins and other APC substrates until APC binds Cdc20 in the subsequent mitosis.

Functions

Separase Activation and the Initiation of Anaphase
Expression of nondegradable cyclin mutants causes fungal, plant, and animal cells to arrest in telophase, i.e., with their sister chromatids separated and transported toward opposite spindle poles in anaphase (Clute and Pines, 1999). However, genetic or biochemical inactivation of the APC blocks mitosis before sister chromatids have been separated in anaphase (Holloway et al., 1993; Irniger et al., 1995; Tugendreich et al., 1995). These observations demonstrated early on that APC activity is required for anaphase but that APC’s role in cyclin proteolysis is not sufficient to explain its function in initiating anaphase. Instead, the results implied that the destruction of at least one other APC substrate is essen-

Table 2. Components of the APC Ubiquitination Pathway

<table>
<thead>
<tr>
<th>Protein</th>
<th>Vertebrates</th>
<th>D.m.</th>
<th>C.e.</th>
<th>S.c.</th>
<th>S.p.</th>
<th>A.n.</th>
<th>Structural Motifs</th>
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<td>E1</td>
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<td>–</td>
<td>Uba1</td>
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<td>Cut4</td>
<td>BIME</td>
<td>Rpn1/2 homology</td>
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<td>Em1</td>
<td>–</td>
<td>–</td>
<td>Rca1</td>
<td>–</td>
<td>–</td>
<td>F box, ZBR</td>
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</table>

D.m., Drosophila melanogaster; C.e., Caenorhabditis elegans; S.c., Saccharomyces cerevisiae; S.p., Schizosaccharomyces cerevisiae; A.n., Aspergillus nidulans. TPRs, tetratrico peptide repeats; ZBR, Zn2+/H11001 binding region; Rpn1/2 are subunits of the 26S proteasome.
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Figure 1. APC-Mediated Proteolysis during the Cell Cycle

Temporal pattern of APC<sup>cd20</sup> and APC<sup>cdh1</sup> activities (A) and of the destruction of some of their substrates (B) during the cell cycle of somatic animal cells. APC<sup>cd20</sup> is activated at the onset of prometaphase (PM) when it initiates the degradation of cyclin A (CycA) and Nek2A. Proteolysis of cyclin B (CycB) and securin (Sec) also depends on APC<sup>cd20</sup> but is delayed until metaphase (M) by the spindle assembly checkpoint. During anaphase (A) and telophase (T), APC<sup>cdh1</sup> is activated and mediates the destruction of additional substrates such as Plk1 and Cdc20. APC<sup>cdh1</sup> is inactivated during mitotic exit, whereas APC<sup>cdh1</sup> remains active until the onset of the next S phase (S). G<sub>2</sub>, G<sub>2</sub> phase; P, prophase.

Figure 2. APC-Mediated Proteolysis during the Cell Cycle

Potential for the initiation of anaphase. This substrate is known as securin.

Expression of nondegradable securin mutants or overexpression of wild-type securin blocks sister chromatid separation (reviewed in Nasmyth, 2001), and, importantly, deletion of securin in budding yeast (which can survive without it at low temperatures) allows sister chromatid separation in the absence of APC activity (Ciosk et al., 1998; Yamamoto et al., 1996). These results imply that APC’s essential role in initiating anaphase must be to destroy securin. Securin binds to a cysteine protease, called separase (reviewed in Nasmyth, 2001). Separase is essential for sister chromatid separation, and its activation depends on securin proteolysis. APC’s essential function in promoting anaphase therefore appears to be the activation of separase.

How then does separase initiate sister chromatid separation? Genetic screens have identified additional genes whose mutation causes precocious sister separation (reviewed by Nasmyth, 2001). Several of these genes encode the subunits of a chromosomal protein complex that is required to hold sister chromatids together from S phase until their separation in anaphase. This complex, called cohesin, is cleaved by separase at the transition from metaphase to anaphase. In budding yeast, this reaction is both required and sufficient to induce sister chromatid separation (Uhmann et al., 1999, 2000), implying that cohesin cleavage directly dissolves cohesion between sisters, allowing their segregation toward opposite spindle poles in anaphase.

Similar although not identical mechanisms appear to regulate sister chromatid separation in higher eukaryotes, including humans. In vertebrates two different pathways dissociate cohesin from mitotic chromosomes (Waizenegger et al., 2000). The bulk of cohesin is removed from chromosomes in prophase and prometaphase by a mechanism that depends on Polo-like kinase but not on APC activation or on cohesin cleavage (Hauf et al., 2001; Losada et al., 1998; Sumara et al., 2002). Indeed, a small amount of cohesin remains on chromosomes until the onset of anaphase, preferentially in centromeric regions (Hauf et al., 2001; Waizenegger et al., 2000). Once separase has been activated through APC-dependent securin proteolysis, these cohesin complexes are cleaved and thereby removed from chromosomes. It is not yet known why vertebrates and possibly other higher eukaryotes remove cohesin from chromosomes in two steps, but it is conceivable that this process is required for chromosome condensation (which is largely absent in budding yeast) or for the separation of sister chromatid arms.

Although securin deletion in budding yeast allows sister separation in the absence of APC activity, recent results suggest that in Xenopus APC’s role in activating separase may not be restricted to securin destruction. By carefully reinvestigating if cyclin B proteolysis is required for anaphase, Stemmann et al. (2001) discovered that high Cdk1 activity prevents sister chromatid separation through inhibitory phosphorylation of separase. This modification alone is sufficient to inhibit separase in the absence of securin binding, possibly explaining why securin-deficient human cells are viable and undergo anaphase with fairly normal timing (Jallepalli et al., 2001). These results suggest that the APC may control separase activation through two distinct mechanisms: through partial cyclin B proteolysis to reduce Cdk1 activity and through securin destruction (Figure 2). This hypothesis could also explain why overexpression of nondegradable cyclin A delays anaphase both in Drosophila and in human cells (reviewed in Su, 2001).

Promoting Anaphase—More Than Sister Separation

Once separase has been activated and sister chromatids have been separated, they are translocated toward the spindle poles through a combination of microtubule shrinkage and the activity of microtubule-dependent motor proteins. Several observations in Xenopus and in budding yeast suggest that APC activity is also critical during this period of mitosis. By systematically screening for proteins that are associated with chromosomes in metaphase but not with chromatids in anaphase, Funabiki and Murray (2000) discovered an APC substrate that is required for chromosome alignment during meta-
APCCdc20 initiates anaphase in Xenopus embryos through at least three distinct mechanisms. (1) APCCdc20 enables activation of the protease separase by mediating the degradation of securin. Active separase separates sister chromatids from each other by cleaving cohesin complexes. (2) APCCdc20 also helps to activate separase by initiating the degradation of cyclin B and other mitotic cyclins. Cyclin degradation inactivates Cdk1 which in turn allows the removal of inhibitory phosphate residues from separase by protein phosphatases (PPases). (3) APCCdc20 also initiates degradation of the kinesin Xkid on chromosome arms. Xkid is a microtubule plus end-directed motor that moves chromosomes away from spindle poles in early mitosis. Xkid degradation, therefore, facilitates the segregation of chromatids toward spindle poles in anaphase.

In early Xenopus embryos, Xkid proteolysis may therefore be required to (Kitamura et al., 1998; Kominami et al., 1998). The same effect is seen, both in fission and budding yeast, if APC subunits are inactivated during G1 (Irniger and Nasmyth, 1997; Kumada et al., 1995). Such mutants inappropriately accumulate mitotic and S phase promoting cyclins in G1 (Irmiger and Nasmyth, 1997; Kominami et al., 1998), and mutation of cyclin genes suppresses the G1 arrest deficiency of APCCdh1 mutants in G1 (Kitamura et al., 1998; Kominami et al., 1998). These observations suggest that in the presence of mating factors or under starvation conditions APCCdh1 is required to prevent cyclin accumulation and the activation of S phase promoting CDKs.

Numerous genetic observations suggest that the G1 function of APCCdh1 is partially redundant with the function of CDK inhibitor proteins, strongly supporting the view that one of APCCdh1’s important functions in G1 is to keep CDKs inactive. For example, the viability of budding yeast strains lacking Cdh1 depends on the CDK inhibitor Sic1 (Schwab et al., 1997; Visintin et al., 1997), and mutation of the fission yeast CDK inhibitor Rum1 causes phenotypes that are virtually identical to the phenotype of Cdh1 mutants (Kominami et al., 1998). Cdh1 also has an important G1 function during Dro-
sophila development. The Cdh1 homolog Fizzy-related is expressed in fly embryos once cells become postmitotic (Sigrist and Lehner, 1997), and, similar to yeast, Fizzy-related is required for arrest in G1. In addition, Fizzy-related is needed to suppress mitosis in certain cell types of the embryo so that these can amplify their DNA content in endoreduplication cycles.

In vertebrate cells, the G1 function of APC

\[ \text{Cdh1} \]

is less well understood. Chicken cells whose Cdh1 alleles have been deleted are viable and apparently proceed through G1 in a normal manner (Sudo et al., 2001). However, Cdh1’s deletion could possibly be compensated by one of several Cdh1 homologs recently discovered in chickens (Wan and Kirschner, 2001). Taken together, the observations made in yeast, Drosophila embryos, and cultured chicken cells imply that APC

\[ \text{Cdh1} \]

is not absolutely essential for the establishment of G1 phases but that it has critical roles in maintaining the G1 state under certain physiological conditions.

In vertebrates, APC

\[ \text{Cdh1} \]

is also present and active in quiescent cultured cells (Brandeis and Hunt, 1996), and it is expressed in a variety of tissues that are predominantly composed of postmitotic cells, such as brain, where it can be detected in the nuclei of differentiated neurons (Gieffers et al., 1999). Although APC

\[ \text{Cdh1} \]

could simply be present in these cells to prevent the accumulation of cyclins and other S phase and mitotic activators, it would be surprising if transcriptional regulation and CDK inhibitor proteins were not sufficient to fulfill this role. It is therefore possible that APC

\[ \text{Cdh1} \]

has additional roles in postmitotic cells that remain to be discovered.

**More G1 and G0 Functions – TGFβ Signaling**

Support for the hypothesis that APC has additional functions in G1 and G0 comes from the recent finding that APC

\[ \text{Cdh1} \]

may regulate TGFβ signaling in animal cells. Signal transduction through the TGFβ pathway has, depending on the cell type and developmental stage, been shown to elicit a variety of biological responses, including inhibition of cell proliferation, differentiation, and apoptosis (reviewed by Massague et al., 2000). Once activated through ligand binding, TGFβ receptors at the plasma membrane phosphorylate cytoplasmic Smad proteins and thereby enable their transport into the nucleus where they control the transcription of specific target genes. For example, TGFβ signaling can activate transcription of the CDK inhibitors p15 and p21 and simultaneously inhibit expression of the growth-promoting transcription factor Myc, resulting in a G1 arrest.

TGFβ signaling is antagonized both by regulated proteolysis of activated Smads and also by several corepressors that repress TGFβ target genes. One of these repressors, called SnoN, is rapidly degraded once TGFβ signaling is activated. Furthermore, overexpression of SnoN can decrease TGFβ signaling, suggesting that SnoN destruction is required for full activation of TGFβ signaling. The ubiquitin ligase Smurf has been implicated in SnoN destruction, but recently it has been reported that SnoN can also be ubiquitinated by APC

\[ \text{Cdh1} \]

and that the APC plays an important role in mediating SnoN proteolysis in response to TGFβ signaling (Stroschein et al., 2001; Wan et al., 2001). These observations suggest that during G1 and G0, APC

\[ \text{Cdh1} \]

helps to restrain cell cycle entry both directly by preventing the accumulation of S phase and mitotic cyclins, and indirectly by allowing TGFβ signaling, which would then inhibit proliferation through transcriptional activation of CDK inhibitor proteins.

**Regulation**

**Mitotic Activation of APC

\[ \text{Cdc20} \]

**

In yeast and in somatic animal cells, Cdc20 is degraded during exit from mitosis in an APC

\[ \text{Cdh1} \]

-dependent manner. Cdc20 therefore has to be resynthesized during S phase and G2 when APC

\[ \text{Cdh1} \]

is inactive and Cdc20 transcription is upregulated (Fang et al., 1998; Kramer et al., 2000; Prinz et al., 1998; Shirayama et al., 1998).
Likewise, it will be important to understand how Emi1 regulates APCCdc20 and how widespread this mechanism is. First experiments suggest that Emi1 can bind to Cdc20 and prevent its interaction with APC substrates but not the binding of Cdc20 to the APC (Reimann et al., 2001b). Emi1 itself is degraded early in mitosis in an APC-independent manner, consistent with its destruction being required for APCCdc20 activation (Reimann et al., 2001a). In Drosophila, a homolog of Emi1, called Rca1, has an important role in keeping APCCdh1 inactive during G2 (see below), but surprisingly there is no evidence yet that Rca1 also regulates APCCdc20. It is therefore possible that mitotic APC phosphorylation and Emi1/Rca1 destruction are of different importance for APCCdc20 activation in different species or cell types or that additional mechanisms of APCCdc20 regulation still remain to be discovered.

**Figure 3. Regulation of APCCdc20**

In prophase or prometaphase, several subunits of the APC are phosphorylated by kinases such as Cdk1 or Plk1. APC phosphorylation increases the binding of Cdc20 to the APC. Cdc20 may activate ubiquitination reactions by recruiting substrates (S) to the APC. In vertebrates, Emi inhibits APCCdc20, possibly by preventing the binding of substrates to Cdc20 until Emi1 itself is degraded early in mitosis. The ability of APCCdc20 to ubiquitinate cyclin B and securin is further delayed by Mad2 and BubR1 until all chromosomes have attached to both poles of the mitotic spindle. During exit from mitosis, Cdc20 is degraded in an APCCdh1-dependent manner.

However, despite Cdc20 accumulation, APCCdc20 is not activated until mitosis, implying that either additional components or modifications are required for APCCdc20 activation or that inhibitors suppress its activity. There is evidence supporting both of these possibilities, although their relative contribution is still poorly understood (Figure 3).

Binding of Cdc20 to the APC and APC activation in mitosis correlates with the phosphorylation of several APC subunits (Rudner and Murray, 2000), and biochemical experiments suggest that these phosphorylation reactions facilitate binding of Cdc20 to the APC (Kramer et al., 2000; Rudner and Murray, 2000; Steineberg et al., 1998; Tang et al., 2001a). In budding yeast, mutation of Cdk1 consensus sites in the APC subunits Cdc27, Cdc16, and Cdc23 largely abolishes APC phosphorylation, reduces Cdc20 binding, and delays exit from mitosis, suggesting that APC phosphorylation by Cdk1 is indeed essential for full activation of APCCdc20 (Rudner and Murray, 2000).

In animal cells, numerous reports have implicated Cdk1 and Plk1 in mitotic APC phosphorylation (reviewed in Zachariae and Nasmyth, 1999), but it is still unclear if this modification is essential for APC activation. Recent work in Xenopus has identified an inhibitor of the APC, called Emi1, whose depletion from Xenopus extracts blocks entry into mitosis because mitotic cyclins fail to accumulate (Reimann et al., 2001a). This observation suggests that Emi1 is required to keep APCCdc20 inactive in interphase and therefore implies that mitotic APC phosphorylation may not be essential for activation of APCCdc20. The identification of in vivo phosphorylation sites and mutational analyses will therefore be required to clarify if and how phosphorylation regulates APCCdc20 in animal cells.

Likewise, it will be important to understand how Emi1 regulates APCCdc20 and how widespread this mechanism is. First experiments suggest that Emi1 can bind to Cdc20 and prevent its interaction with APC substrates but not the binding of Cdc20 to the APC (Reimann et al., 2001b). Emi1 itself is degraded early in mitosis in an APC-independent manner, consistent with its destruction being required for APCCdc20 activation (Reimann et al., 2001a). In Drosophila, a homolog of Emi1, called Rca1, has an important role in keeping APCCdh1 inactive during G2 (see below), but surprisingly there is no evidence yet that Rca1 also regulates APCCdc20. It is therefore possible that mitotic APC phosphorylation and Emi1/Rca1 destruction are of different importance for APCCdc20 activation in different species or cell types or that additional mechanisms of APCCdc20 regulation still remain to be discovered.

**Regulation of APCCdc20 by the Spindle Assembly Checkpoint**

The hypothesis that APCCdc20 is activated in early mitosis either by phosphorylation or by Emi1/Rca1 degradation can explain why APCCdc20 is inactive in interphase, but it does not explain how the activation of APCCdc20 during mitosis is timed in a way that enables the APC to precisely control late mitotic events such as anaphase and exit from mitosis. It is well established that mitotic A-type cyclins and the kinase Nck2A are degraded during prometaphase in an APC- and Cdc20-dependent manner, indicating that APCCdc20 is already active early in mitosis (Dawson et al., 1995; den Elzen and Pines, 2001; Geley et al., 2001; Hames et al., 2001; Sigrist et al., 1995). In contrast, other APC substrates such as securin and cyclin B are stable until the beginning of metaphase. Their degradation is initiated by APCCdc20 only once all chromosomes have attached to both poles of the mitotic spindle. This timing mechanism ensures that sister chromatic cohesion is not dissolved by separase before spindle assembly has been completed, which could otherwise lead to chromatic missegregation and the formation of aneuploid daughter cells.

The biochemical pathway that delays securin and cyclin B proteolysis until all chromosomes are bipolarly attached is called the spindle assembly checkpoint (reviewed by Amon, 1999). Remarkably, a single unattached kinetochore is sufficient to delay the separation of all the chromatids in a mitotic cell (Rieder et al., 1994). The checkpoint is thought to measure tension that is created at kinetochores once both sister chromatids are attached to opposite poles (Li and Nicklas, 1995; Stern and Murray, 2001), but there may also be mechanisms that measure the attachment of spindle microtubules to kinetochores directly, independent of tension (Skoufias et al., 2001; Waters et al., 1998). How activation of the checkpoint specifically inhibits the ability of APCCdc20 to ubiquitinate securin and cyclin B is still poorly understood. Two known checkpoint proteins, Mad2 and BubR1, can inhibit APCCdc20 in vitro, but it is unclear if Mad2 and BubR1 collaborate to inhibit APCCdc20 or if Mad2 and BubR1 are activated by different branches of the spindle assembly checkpoint.

Mad2 can either bind to the checkpoint protein Mad1 or to Cdc20 but not simultaneously to both (Hwang et al., 1998; Sironi et al., 2001). The binding of Mad2 to Mad1 recruits Mad2 specifically to unattached kineto-
chores, induces a major conformational change in Mad2, and appears to facilitate the binding of Mad2 to Cdc20 (Chen et al., 1996; Luo et al., 2002; Sironi et al., 2001). These observations suggest that unattached kinetochores recruit Mad1 which then can bind Mad2 and convert it into a form that can interact with Cdc20. How Mad2-Cdc20 complexes then inhibit the APC is not known. In vitro Mad2 can inhibit the association of Cdc20 with the APC (Reimann et al., 2001b; Tang et al., 2001a), suggesting that Mad2 could inhibit APC by titrating out Cdc20. However, it is unclear if a single unattached kinetochore, which is sufficient to delay anaphase, could inactivate enough Cdc20 to prevent cyclin B and securin destruction. It is easier to envision that Mad2-Cdc20 complexes inhibit APC function in a dominant manner. Consistent with this hypothesis, Mad2 can indeed be specifically detected in mitotic APC immunoprecipitates (Kallio et al., 1998; Wassmann and Benezra, 1998), Mad2 can also slow down the dissociation of Cdc20-substrate complexes in vitro (Pfleger et al., 2001b), raising the possibility that Mad2 interferes with some step during substrate ubiquitination.

Recent biochemical fractionation experiments showed that only a complex containing Mad2, Cdc20, BubR1, and Bub3 is able to efficiently inhibit APC\textsuperscript{Cdc20} in vitro, whereas fragments that contain Mad2 molecules which are not part of this mitotic checkpoint complex (MCC) have no inhibitory effect (Sudakin et al., 2001). Experiments with recombinant proteins suggest that BubR1 is largely responsible for the inhibitory activity of MCC because this protein alone can bind to Cdc20, prevent its interaction with the APC, and inhibit securin and cyclin B ubiquitination in vitro (Tang et al., 2001a). In the future it will be important to understand if MCC inhibits Cdc20 via both its Mad2 and BubR1 subunits and if active Mad2 exists exclusively as part of MCC, or if independent checkpoint complexes exist. Likewise, it will be crucial to understand how APC\textsuperscript{Cdc20} is able to ubiquitinate cyclin A but not B-type cyclins and securin in the presence of an activated spindle assembly checkpoint.

**Mitotic Exit—Switching from APC\textsuperscript{Cdc20} to APC\textsuperscript{Cdh1}**

During most of mitosis, Cdh1 is phosphorylated by CDKs and thereby kept inactive. At the end of mitosis, Cdh1 is dephosphorylated and thus able to bind and activate the APC (Blanco et al., 2000; Jaspersen et al., 1999; Kramer et al., 2000; Listovsky et al., 2002; Lukas et al., 1999; Yamaguchi et al., 2000; Zachariae et al., 1998a). APC\textsuperscript{Cdh1} then ubiquitinates Cdc20 and thereby inactivates APC\textsuperscript{Cdc20} (Pfleger and Kirschner, 2000; Prinz et al., 1998; Shirayama et al., 1998). Around the same time, one of the E2 enzymes that collaborate with the APC, UbcH10, is degraded (Yamanaka et al., 2000), but the physiological consequence of this reaction is not yet known. Neither Cdc20 proteolysis nor the degradation of UbcH10 is essential for activation of APC\textsuperscript{Cdc20} in the catalytic component of the APC. The activation of APC\textsuperscript{Cdh1} is not triggered by a decrease in the amount of APC\textsuperscript{Cdc20}, but rather by the simultaneous accumulation of the APC proteins Cdh1 and Sic1. This mechanism ensures that cells never exit from mitosis before anaphase has been initiated or under conditions where chromosomes would not be segregated correctly between mother and daughter cells.

In yeast, activation of both APC\textsuperscript{Cdh1} and Sic1 depends on the protein phosphatase Cdc14, which is activated in anaphase and telophase, APC\textsuperscript{Cdh1} is activated once degradation of B-type cyclins has been initiated by APC\textsuperscript{Cdc20}, raising the simple possibility that lowering Cdk1 activity may be sufficient to allow Cdh1 dephosphorylation by constitutively active protein phosphatases. However, recent work has revealed that regulation of Cdh1 dephosphorylation is more complex (Figure 4). In budding yeast, APC\textsuperscript{Cdc20} initiates mitotic cyclin degradation but is unable to complete it (Baumer et al., 2000; Yeong et al., 2000). A fraction of mitotic cyclins is protected from destruction by an unknown mechanism until APC\textsuperscript{Cdh1} is activated at the end of mitosis. The activation of APC\textsuperscript{Cdh1} in turn depends on the prior activation of APC\textsuperscript{Cdc20} and on the correct positioning of the mitotic nucleus between the mother and the future daughter cell (reviewed by Bardin and Amon, 2001). Once these events have occurred, CDK inactivation is completed by activation of APC\textsuperscript{Cdh1} and by the simultaneous accumulation of the CDK inhibitor Sic1. This mechanism ensures that cells never exit from mitosis before anaphase has been initiated or under conditions where chromosomes would not be segregated correctly between mother and daughter cells.
Cdh1, Sic1, and the transcription factor Swi5, all of which contribute to CDK inactivation (Shou et al., 1999; Visintin et al., 1998). Cdh1 dephosphorylation activates APC^{Cdh1}, whereas Sic1 dephosphorylation prevents its recognition by SCF ubiquitin ligases and therefore results in Sic1 stabilization. Sic1 accumulation is also promoted by Swi5 dephosphorylation which allows transport of Swi5 into the nucleus where it stimulates Sic1 transcription. Once accumulated, Sic1 inactivates mitotic CDKs through direct binding and might therefore collaborate with APC^{Cdh1} in promoting exit from mitosis.

Activation of the Cdc14 phosphatase is controlled by at least two mechanisms. In anaphase, Cdc14 is transiently released from the nucleolus by the FEAR pathway (Stegmeier et al., 2002). This pathway depends on separate activation, but what the critical separate substrates are and how the FEAR pathway dissociates Cdc14 from Cfl1/Net1 are not yet known. Maintenance of Cdc14 activity and exit from mitosis depend on a second pathway, called the mitotic exit network (MEN). The MEN represents a signaling cascade that consists of the GTPase Tem1, two proteins that regulate Tem1 (a GTPase activating protein and a guanine-nucleotide exchange factor), and several protein kinases (reviewed in Bardin and Amon, 2001). Activation of the MEN is controlled by multiple signals, including the correct positioning of the nucleus between the mother and the future daughter cell, which brings the GTPase Tem1 into close proximity to its GTPase activating protein, Lte1 (Bardin et al., 2000). Tem1 is concentrated at the spindle pole that will move into the daughter cell, where Lte1 is also enriched at the cortex. Tem1 is therefore only fully activated once the Tem1-containing spindle pole has moved into the daughter cell. Activation of Tem1 then promotes release of Cdc14 from the nucleolus and thereby initiates exit from mitosis.

Homologs of Cdc14 also exist in other eukaryotes, but whether they are required for Cdh1 dephosphorylation and mitotic exit is unknown. In fission yeast, Cdc14 is regulated by a signaling cascade similar to the MEN in budding yeast, called the septation initiation network (SIN), but this pathway appears to control CDK inactivation by promoting inhibitory tyrosine phosphorylation on Cdk1, not by activating cyclin protein synthesis (reviewed by Bardin and Amon, 2001). In higher eukaryotes, it has been shown that a homolog of Cdc14 can dephosphorylate Cdh1 in vitro (Bembelen and Yu, 2001), but it is not yet known if this enzyme is required for APC activation in vivo.

**Inactivation of APC^{Cdh1} in S, G2, and Early Mitosis**

APC^{Cdh1} has to be inactivated at the G1-S transition to allow the reaccumulation of APC substrates that are needed for entry into S phase or later for entry into mitosis. For example, to allow DNA replication, S phase-promoting cyclins have to accumulate, such as cyclin A in vertebrates and Clb5 and Clb6 in budding yeast. Phosphorylation of Cdh1 appears to be required for its inactivation in all organisms studied so far. In budding yeast, G1-CDKs, whose cyclin subunits are not APC substrates, are thought to initiate Cdh1 phosphorylation, but S phase-promoting CDKs complete and maintain Cdh1 phosphorylation (Amon et al., 1994; Huang et al., 2001). In *Drosophila* and mammalian cells, cyclin E-Cdk2 and cyclin A-Cdk2 have been reported as the major Cdh1 kinases, respectively (Lukas et al., 1999; Sigrist and Lehner, 1997).

Dephosphorylation of Cdh1 dissociates it from the APC but also might target it for degradation. Cdh1 protein levels decrease during S phase in vertebrate cells and fission yeast when Cdh1 is phosphorylated, and nonphosphorylatable mutants of fission yeast Cdh1 are stabilized in S phase (Blanco et al., 2000; Kramer et al., 2000; Lukas et al., 1999; Yamaguchi et al., 2000). How Cdh1 is degraded is unknown, but it is conceivable that SCF complexes are involved because the recognition of substrates by these ubiquitin ligases typically depends on substrate phosphorylation (reviewed by Deshaies, 1999).

Although Cdh1 phosphorylation is required for APC^{Cdh1} inactivation, the recent identification of Rca1/Emi1 as an APC inhibitor suggests that phosphorylation alone is not sufficient to prevent APC-mediated proteolysis in S and G2 phase. *Drosophila* Rca1 was initially characterized as a regulator of cyclin A whose mutation resulted in a G2 arrest similar to the one caused by mutation of cyclin A (Dong et al., 1997). More recently, Grosskortenhuis and Sprenger (2002) showed that mitotic cyclins are prematurely degraded in Rca1 mutants and that cyclin degradation mediated by the *Drosophila* Cdh1 ortholog Fizzy-related can be inhibited by Rca1. These observations and the earlier finding that Rca1 mutants arrest in G2 indicate that Rca1 function is required to keep APC^{Cdh1} inactive during G2, possibly because animal cells do not contain enough CDK activity in G2 to inactivate Cdh1 by phosphorylation alone.

Similar although not identical results have been obtained with Emi1 in vertebrate cells. Emi1 was initially found to inhibit APC^{Cdk2} in Xenopus extracts (see above), but more recently inhibition of APC^{Cdh1} has also been reported (Reimann et al., 2001b). Emi1 is able to inactivate purified APC, possibly by binding to Cdc20 and Cdh1 and preventing their interaction with APC substrates. Since Emi1 interacts with the Skp1 subunit of SCF ubiquitin ligases via an F box (Reimann et al., 2001a), a sequence element that is typically found in substrate adaptors of the SCF, it will be interesting to test if Emi1 inhibits Cdh1 not only by direct binding but also by targeting it for ubiquitination by SCF complexes.

**Structure and Mechanism of the APC**

Although it is clear that the APC is essential for the multiubiquitination of its substrates, very little is known about how the APC supports these reactions in a mechanistic sense. In operational terms, the APC fulfills the role of a ubiquitin ligase, i.e., its presence is required for the transfer of ubiquitin residues from E2 enzymes to substrates. This reaction results in the formation of an isopeptide bond between the C terminus of ubiquitin and the epsilon amino group of a lysine residue in the substrate. In subsequent reaction cycles, lysine residues within the attached ubiquitin residues can also serve as acceptor sites, resulting in the assembly of a mult ubiquitin chain which is thought to function as a recognition signal for the 26S proteasome.

Based on sequence homologies and biochemical
properties, two types of ubiquitin ligases can be distinguished. HECT domain proteins form covalent ubiquitin thioesters before they transfer ubiquitin residues to substrates. All other known ubiquitin ligases, including the APC, are characterized by the presence of a RING finger, a protein domain that is stabilized by the coordination of two atoms of zinc. RING fingers are not known to form ubiquitin thioester intermediates but have been shown to directly bind to E2 enzymes (cited in Gmachl et al., 2000). When mixed with E1 and E2 enzymes, isolated purified RING finger proteins are able to support ubiquitination reactions in vitro (Lorick et al., 1999). RING finger proteins therefore appear to have an important role in the catalysis of ubiquitination reactions, but it remains unknown whether they directly participate in catalysis or just facilitate it by generating proximity between E2 enzymes and substrates. Support for the first possibility has been provided recently by Tang et al. (2001b), who reported that high concentrations of Zn²⁺ ions alone are able to support ubiquitination reactions in the absence of a RING finger protein.

Among RING finger-containing ubiquitin ligases, the APC appears to be distantly related to SCF complexes because its Apc2 subunit is partially homologous with the cullin subunit of SCF (Yu et al., 1998; Zachariae et al., 1998b). In SCF the C-terminal domain of the cullin subunit binds to the RING finger subunit, whereas the cullin N terminus interacts with two other proteins that can bind to substrates (Deshaies, 1999; Zheng et al., 2002). Culls may therefore help to bring substrates, RING finger proteins, and E2 enzymes into close proximity. Apc2 may have a similar function because it binds to APC’s RING finger subunit, Apc11, which in turn is able to bind E2 enzymes and to support ubiquitination reactions in vitro (Gmachl et al., 2000; Leverson et al., 2000; Tang et al., 2001b). However, it remains unknown how substrates are recruited to Apc2-Apc11.

The APC is unusual among ubiquitin ligases with respect to its subunit complexity. Eleven to twelve core subunits have been identified in the yeast and the human APC (Gmachl et al., 2000; Grossberger et al., 1999; Yu et al., 1998; Zachariae et al., 1998b). This complexity is surprising because many other ubiquitin ligases are only composed of one or a few subunits, implying that ubiquitin ligase activity does not necessarily depend on multiple subunits. It has been discussed that the multitude of APC subunits could reflect the complexity of APC’s regulation, but it is also not clear if a dozen subunits would really be required to subject APC activity to tight cell cycle control. It therefore remains a big mystery as to why the APC is composed of so many subunits and what their individual functions are.

Structure
Cryo-electron microscopy of purified human interphase APC has yielded a low resolution model of the APC that shows an asymmetrically shaped particle with an internal cavity (Gieffers et al., 2001). This cavity would be large enough to accommodate cofactors such as E2 enzymes or substrates of the APC. It will therefore be interesting to analyze whether ubiquitination reactions take place inside this cavity and whether the activity of the APC can be regulated by spatially controlling the access of substrates to catalytically important subunits within this cavity. Higher resolution structures and the mapping of individual subunits in the cryo model will be important to test these ideas and to obtain insight into how this complex may ubiquitinate substrates.

So far only the crystal structures of the small APC subunit Apc10/Doc1 and of a fragment of Apc2 have been reported (Au et al., 2002; Wendt et al., 2001; Zheng et al., 2002). Apc10/Doc1 is characterized by a “Doc” domain that is also found in several other putative ubiquitin ligases of both the HECT and the RING finger type (Grossberger et al., 1999; Komimani et al., 1998). Apc10/Doc1 may therefore have a function that is common to some ubiquitin ligases. The structures of Apc10/Doc1 have revealed that the Doc domain is virtually identical to the jellyroll domain previously found in a variety of eukaryotic and bacterial proteins. In all known cases the jellyroll domain binds ligands, such as sugars, nucleotides, phospholipids, nucleic acids, or proteins. By analogy, Apc10/Doc1’s Doc domain may also bind an unidentified ligand. This reaction could be important for APC function because a mutation in budding yeast Apc10/Doc1 that would be predicted to destabilize the putative ligand binding pocket renders the yeast APC temperature sensitive (Hwang and Murray, 1997).

Do Cdc20 and Cdh1 Recruit Substrates to the APC?
The role of Cdc20 and Cdh1 in regulating the APC was first discovered in yeast and Drosophila where mutation of these proteins stabilizes APC substrates, and Cdc20 and Cdh1 overexpression has the opposite effect (Dawson et al., 1995; Schwab et al., 1997; Sigrist et al., 1995; Sigrist and Lehner, 1997; Visintin et al., 1997). Interestingly, budding yeast Cdc20 and Cdh1 appear to activate the APC in a substrate specific manner; for example, Cdc20 being required for degradation of the securin Pds1 but not for complete proteolysis of Cb2 which in turn depends on Cdh1 (Visintin et al., 1997). This observation suggested early on that Cdc20 and Cdh1 may activate the APC by recruiting specific substrates to the ubiquitin ligase, at least in budding yeast (in Drosophila and Xenopus embryos that lack Cdh1, APCCDc20 is clearly able to ubiquitinate both securin and mitotic cyclins).

Recent support for the hypothesis that Cdc20 and Cdh1 recruit substrates to the APC comes from two observations. First, chicken cells were found to contain four homologs of Cdh1 which in vitro all activated the APC toward different substrates, consistent with the possibility that these proteins recruit different substrates to the APC (Wan and Kirschner, 2001). Interestingly, these Cdh1 homologs are differentially expressed in chicken tissues, also lending further support to the hypothesis that the APC may have unidentified functions in differentiating cells. Second, several groups found that APC substrates can bind to Cdc20 and Cdh1 (Burton and Solomon, 2001; Hilioti et al., 2001; Ohtoshi et al., 2000; Pfleger et al., 2001a; Schwab et al., 2001; Sorensen et al., 2001). Importantly, Schwab et al. (2001) observed that the ability of overexpressed Cdc20 and Cdh1 to destabilize specific substrates in vivo corresponds to their ability to bind to these substrates in vitro. Furthermore, Pfleger et al. (2001a) showed that N-terminal fragments of Cdc20 and Cdh1 that can bind APC substrates are also able to inhibit the degradation of APC substrates in Xenopus extracts, suggesting that...
the in vitro binding reactions are relevant for APC-mediated ubiquitination reactions.

Although these data suggest that Cdc20 and Cdh1 bind to APC substrates, there are also several conflicting and unexplained observations. For example, Schwab et al. (2001) observed that Cdh1 could bind to the mitotic cyclin Clb2 even when Clb2’s D box had been deleted, despite the fact that Cdh1-induced Clb2 degradation in vivo is strictly dependent on the D box. This could imply that in vitro binding is not always an accurate correlate of the binding interaction that occurs in vivo or that the D box is not actually required for binding of substrates to Cdh1 but for their subsequent ubiquitination. Likewise, it is not clear which parts of Cdc20/Cdh1 are required for substrate binding. Sørensen et al. (2001) showed that cyclin A can bind to a conserved sequence element in the C-terminal WD40 domain of Cdh1, whereas Pfieger et al. (2001a) proposed an important role for the N termini of Cdc20 and Cdh1. However, if Cdc20’s and Cdh1’s N-terminal domains were their only determinants of substrate specificity, it would be difficult to explain how the recently identified homologs of Cdh1 in chicken cells could confer substrate specificity to the APC, because all of these homologs are almost identical in their N-terminal domains (Wan and Kirschner, 2001).

Conclusions

APC’s mitotic functions and regulation are now understood in some detail, but several mysteries remain; for example, how the APC is able to ubiquitinate cyclin A early in mitosis without touching other substrates such as cyclin B and securin. Also, the lists of APC’s cell cycle substrates and of pathways that modulate its activity are likely to grow in the future. Bigger surprises, however, may come from areas other than cell cycle research, which may uncover unexpected roles for the APC in nonproliferating cells and tissues. What we are still missing almost entirely is a mechanistic understanding of how the APC mediates ubiquitination reactions. Although this is also far from clear for other RING finger-containing ubiquitin ligases, the APC represents a particularly puzzling example due to its unusual size and still unexplained subunit complexity. The lack of mechanistic insight is in part due to the scarcity of this complex, which makes its conventional purification in large scale difficult, and to its subunit complexity, which has so far prevented successful in vitro reconstitutions from recombinant proteins. Therefore, many more years may pass before we will fully understand how the APC works and how exactly Hunt’s cyclin is degraded in mitosis.

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