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THE ROYAL SOCIETY

Control of mitotic transitions by the anaphase-promoting complex

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Proteolysis controls key transitions at several points in the cell cycle. In mitosis, the activation of a large ubiquitin–protein ligase, the anaphase-promoting complex (APC), is required for anaphase initiation and for exit from mitosis. We show that APC is under complex control by a network of regulatory factors, CDC20, CDH1 and MAD2. CDC20 and CDH1 are activators of APC; they bind directly to APC and activate its cyclin ubiquitination activity. CDC20 activates APC at the onset of anaphase in a destruction box (DB)-dependent manner, while CDH1 activates APC from late anaphase through G1 with apparently a much relaxed specificity for the DB. Therefore, CDC20 and CDH1 control both the temporal order of activation and the substrate specificity of APC, and hence regulate different events during mitosis and G1. Counteracting the effect of CDC20, the checkpoint protein MAD2 acts as an inhibitor of APC. When the spindle-assembly checkpoint is activated, MAD2 forms a ternary complex with CDC20 and APC to prevent activation of APC, and thereby arrests cells at prometaphase. Thus, a combination of positive and negative regulators establishes a regulatory circuit of APC, ensuring an ordered progression of events through cell division.

Keywords: anaphase-promoting complex (APC); spindle-assembly checkpoint; protein degradation; CDC20; CDH1; MAD2

1. INTRODUCTION

It has been recently appreciated that though the mechanisms of intracellular protein degradation are very general, they can be used to regulate specific cellular events in the cell cycle, signal transduction, transcription, development and in the immunoresponse in vertebrates (Glotzer *et al.* 1991; Holloway *et al.* 1993; Jiang & Struhl 1998). Proteolytic control in the cell cycle was first established as part of the regulation of cyclin-dependent kinases (King *et al.* 1996*a*). In this initial view, the purpose of the mitotic degradation pathway was to inhibit the mitotic cyclin B/Cdc2 kinase; a separate G1/S degradation machinery activated the S-phase kinase by degrading an inhibitor of that kinase (Glotzer *et al.* 1991; Schwob *et al.* 1994; Bai *et al.* 1996; Feldman *et al.* 1997). It is now clear that both proteolytic pathways have more substrates than just kinases and kinase inhibitors. Each pathway controls several key transitions in the cell cycle. For example, the mitotic degradation pathway not only controls exit from mitosis by degrading B-type cyclins, but also triggers the metaphase-to-anaphase transition by degrading the anaphase inhibitors Pds1p and Cut2p (figure 1) (Holloway *et al.* 1993; Cohen-Fix *et al.* 1996; Funabiki *et al.* 1996; Yamamoto *et al.* 1996*a*,*b*; Ciosk *et al.* 1998).

The mitotic degradation pathway shares overall features with other degradation systems. Pds1p/Cut2p and cyclin B are degraded in a ubiquitin-dependent process, in which a ubiquitin-activating enzyme (E1) activates and transfers the ubiquitin protein to a ubiquitin-conjugating enzyme (E2). E2, together with a ubiquitin–protein ligase, conjugates ubiquitin to these target proteins, which are subsequently degraded by the proteasome. In vertebrates, two ubiquitin-conjugating enzymes, Ubc4 and Ubcx/E2-C, work equally well in cyclin ubiquitination *in vitro* and probably carry out redundant function *in vivo* (King *et al.* 1995; Aristarkhov *et al.* 1996; Yu *et al.* 1996). The cell-cycle-regulated component in the mitotic degradation pathway is a 20S ubiquitin–protein ligase, the anaphase-promoting complex (APC), also known as the cyclosome; APC is named for its role in control of the metaphase-to-anaphase transition (King *et al.* 1995). In vertebrates, APC consists of at least eight subunits, named APC1–APC8, most of which are highly conserved through evolution. Several subunits, APC1 (BimE), APC3 (CDC27), APC6 (CDC16) and APC8 (CDC23), have also been identified through genetic studies in yeast and in *Aspergillus*, and mutations in any of these subunits arrest cells at metaphase, consistent with their role in anaphase initiation (Hershko *et al.* 1994; Irniger *et al.* 1995; King *et al.* 1995; Sudakin *et al.* 1995; Tugendreich *et al.* 1995; Peters *et al.* 1996; Zachariae & Nasmyth 1996; Zachariae *et al.* 1996, 1998*b*; Yu *et al.* 1998).

Temporal control of APC activity is through post-translational mechanisms, which specify overall enzymatic activity and substrate selection. In *Xenopus* early embryonic cell division, APC is active in mitosis, but has only a basal level of activity in interphase (King *et al.* 1995). In mammalian tissue culture cells and in yeast, APC becomes active at the onset of anaphase and its activity persists through most of G1. The APC activity is low in S, G2 and in early mitosis, as assayed by cyclin ubiquitination (Amon *et al.* 1994; Brandeis & Hunt 1996;

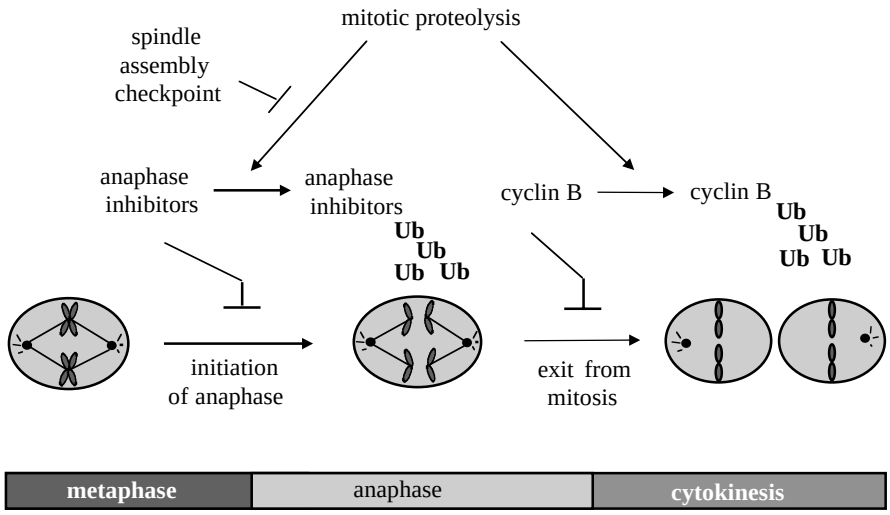

Figure 1. Mitotic proteolysis controls both the metaphase-to-anaphase transition and the exit from mitosis.

Fang *et al.* 1998*b*). During mitosis, the APC pathway degrades multiple substrates, such as Pds1p/Cut2p and cyclin B, at different stages of mitosis. A major goal of our current studies, as described in this report, is to understand what controls the overall activation of APC at the onset of anaphase and what determines the timing of substrate degradation through mitosis.

Overlaying substrate selection during the mitotic cycle are surveillance mechanisms to ensure that cell cycle progression is tied to the integrity of the genome and the fidelity of the chromosome separation (Elledge 1996). A spindle-assembly checkpoint, for example, is thought to allow anaphase to initiate only after monitoring the attachment of kinetochores to the mitotic spindle (Murray 1994, 1995; Rudner & Murray 1996; Nicklas 1997). As activation of APC is required for the metaphase-to-anaphase transition, APC appears to be a key target for the checkpoint intervention (figure 1). In this paper, we will report our recent progress in understanding the cell cycle regulation of APC and the mechanism of checkpoint-mediated inhibition of APC.

2. ACTIVATION OF APC IN MITOSIS

(a) *Activators of APC*

APC purified from interphase extracts of *Xenopus* eggs shows little activity against the cyclin B substrate, while mitotic APC causes rapid and quantitative ubiquitination. To identify activators of APC in mitosis, we fractionated mitotic *Xenopus* egg extracts and found two activities that can independently activate interphase APC (Fang *et al.* 1998*b*). One activity requires ATP and is likely to be a kinase. Further purification indicated that this activity co-fractionates with the polo-like kinase (Plk1), consistent with a recent report on direct phosphorylation and activation of APC by Plk1 (Kotani *et al.* 1998). The other activity activates APC in the absence of ATP and is due to binding of a regulatory factor to APC. Further purification identified this activity as the vertebrate homologue of yeast Cdc20p. We assume that *in vivo* both activities are required.

There are two members of the CDC20 protein family in yeast: Cdc20p and Cdh1p/Hct1p, both of which contain seven repeats of the WD40 motif. *Drosophila* has a similar pair of proteins, called fizzy and fizzy-related, while vertebrates also have homologues of CDC20 and CDH1. We will follow the vertebrate nomenclature. Genetic studies in yeast and *Drosophila* indicate that CDC20 and CDH1 are both involved in activation of the mitotic degradation pathway (Schwab *et al.* 1997; Sigrist & Lehner 1997; Visintin *et al.* 1997). For example, ectopic expression of either Cdc20p or Cdh1p in yeast activates the degradation machinery independent of the cell cycle stage. We showed that CDC20 and CDH1 are activators of APC (Fang *et al.* 1998*b*). The recombinant human CDC20 and CDH1 directly bind to interphase APC and activate its cyclin ubiquitination activity (figure 2). Similarly, incubation of mitotic APC with CDC20 and CDH1 also enhance its ubiquitination activity, suggesting that mitotic APC contains substoichiometric amounts of these activators and that CDC20 and CDH1 are limiting factors in the activation of APC.

A few subunits of APC are phosphorylated during mitosis (King *et al.* 1995; Peters *et al.* 1996). Binding studies indicate that both CDC20 and CDH1 have a higher affinity for mitotic phosphorylated APC than for interphase APC, pointing to a potential function of phosphorylation of APC during mitosis (Fang *et al.* 1998*b*). We have also identified a kinase activity (Plk1) in the mitotic extracts that can activate interphase APC, presumably by phosphorylating APC and enhancing its association with the small amount of CDC20 present in the kinase fractions. It has been previously reported that dephosphorylation of mitotic APC inactivates its ubiquitination activity, suggesting a critical role of phosphorylation in APC activation (Hershko *et al.* 1994; King *et al.* 1995; Lahav-Baratz *et al.* 1995; Peters *et al.* 1996). However, we show that a large excess of CDC20 or CDH1 is sufficient to activate APC independent of the phosphorylation state of APC. We speculate that at limiting amounts of CDC20 and CDH1, dephosphorylation of mitotic APC might cause dissociation of the bound activators and therefore inactivation of mitotic APC (Fang *et al.* 1998*b*). Thus, phosphorylation of APC in the presence of a low concentration of CDH1 and CDC20 could be limiting and required.

CDC20 and CDH1 also contribute to substrate selection by APC (Fang *et al.* 1998*b*). It had been shown previously

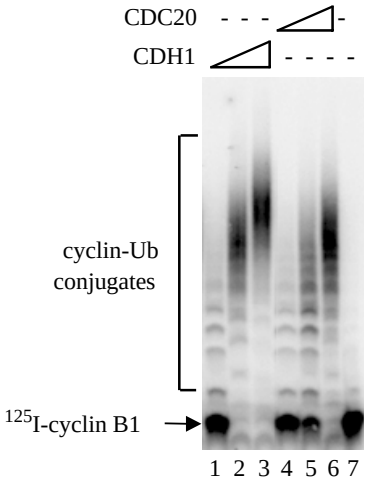

Figure 2. Activation of APC by CDC20 and CDH1. Recombinant human CDC20 and CDH1 were expressed in insect Sf9 cells and purified to homogeneity. 0.5 μg (lanes 1 and 4), 1 μg (lanes 2 and 5), 5 μg (lanes 3 and 6) and 0 μg (lane 7) of the recombinant proteins were incubated with interphase APC immunopurified from *Xenopus* egg extracts. The APC beads were then washed and assayed for ability to ubiquitinate a ^{125}I-labelled N-terminal fragment of *Xenopus* cyclin B1.

that APC recognizes a nine-amino-acid sequence called the destruction box (DB) in the N-terminal region of the mitotic cyclins (Glotzer *et al.* 1991; King *et al.* 1996*b*). Though both CDC20- and CDH1-activated APC ubiquitinate cyclin B1 efficiently, they have different requirements for the DB. CDC20-associated APC is strictly DB-specific. In contrast, APC activated by CDH1 supports ubiquitination of both the wild-type and DB deletion mutant of cyclin B1, and therefore, has a much relaxed requirement for the DB. Indeed, certain CDH1-specific substrates identified so far, such as vertebrate CDC20, Plk1 and ARK2 (see below), do not seem to contain the DB. Although the exact structural element recognized by CDH1-associated APC remains unclear, the differential substrate selection by CDC20 and CDH1 coupled with their cell-cycle stage-specific association with APC (see below) could partly explain degradation of different substrates at different times of the cell cycle (Schwab *et al.* 1997; Visintin *et al.* 1997; Fang *et al.* 1998*b*).

(b) *Regulation of APC activation*

We have found that the expression of CDC20 and CDH1 is regulated during the cell cycle in HeLa cells. Transcripts of both genes accumulate in G2, peak in mitosis and disappear in G1. The level of the CDC20 protein follows a similar pattern, accumulating from late G2 through mitosis and dropping drastically in early G1 (figure 3*a*,*b*). Therefore, the CDC20 protein is most likely unstable. Indeed, CDC20 is degraded by the CDH1-associated APC as cells exit from mitosis (see below). In contrast, CDH1 is a stable protein whose level does not vary significantly through the cell cycle (Fang *et al.* 1998*b*; Prinz *et al.* 1998; Shirayama *et al.* 1998).

The variation in CDC20 protein levels is not sufficient to explain the regulation of APC activity. The association of CDC20 and CDH1 with APC is also regulated independently. CDC20 becomes associated with APC at

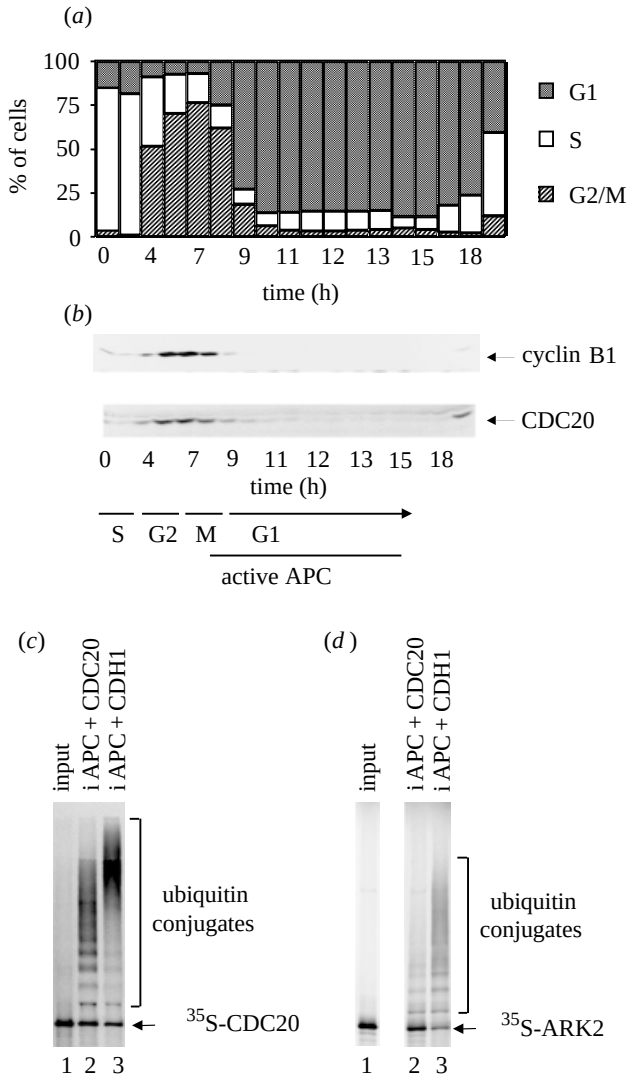

Figure 3. CDC20 and ARK2 are substrates of CDH1-activated APC. (*a*, *b*) Cell-cycle regulation of the CDC20 protein. HeLa cells were synchronized at the G1–S boundary by a double thymidine block, and cells were collected at indicated time-points after being released from the arrest. Cell-cycle stage were determined by FACS analysis (*a*), and the level of cyclin B1 and CDC20 proteins were determined by Western blot analysis (*b*). Like cyclin B1, CDC20 is an unstable protein. (*c*, *d*) CDC20 and ARK2 can be efficiently ubiquitinated by the CDH1-activated APC. Interphase APC immunopurified from *Xenopus* egg extracts was activated by incubating with recombinant human CDC20 (lane 2) and CDH1 (lane 3). The APC beads were then washed and assayed for ubiquitination of ^{35}S-labelled CDC20 and ARK2.

metaphase, long after its accumulation in G2 through prophase. Its association is temporally coincident with the activation of APC at the metaphase-to-anaphase transition. APC, activated by CDC20, then triggers the anaphase initiation by degrading the anaphase inhibitors Pds1p/Cut2p. Later in mitosis, CDC20 dissociates from APC, and CDH1, which is present throughout the cell cycle, binds to and activates APC, leading to a change of APC substrate specificity. A small amount of CDH1 remains associated with APC in G1 and we assume that CDH1 is responsible for maintaining APC activity in G1 (Fang *et al.* 1998*b*).

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Table 1. *APC substrates*

APC substrates	functions	timing of degradation
Pds1p/Cut2p	anaphase inhibitors	metaphase → anaphase
mitotic cyclins	activators of Cdc2 kinase	late anaphase → G1
CDC20	activator of APC	late anaphase
Ase1p	spindle elongation	late anaphase
geminin	inhibitor of DNA replication	late anaphase
Plk1/Cdc5p	mitotic kinases	G1
ARK2/aurora/Ipl1p	mitotic kinases	G1

It therefore appears that the binding of CDC20 and CDH1 to APC are independently regulated to allow precise control of APC activity in the cell cycle. Although the level of the CDC20 protein peaks at early mitosis, its association with APC does not occur until metaphase. Similarly, although the CDH1 protein is present throughout the cell cycle, it only becomes associated with APC from late mitosis through G1. Little is known about what controls the association between CDC20 and APC at the metaphase-to-anaphase transition and what triggers the dissociation of CDC20 from APC later in the mitosis. Recent findings in yeast provide some insight into the trigger for CDH1 binding to APC in late mitosis. CDH1 is phosphorylated by the Cdc2 kinase during early mitosis, and dephosphorylation of CDH1 in late mitosis leads to its binding to APC (Zachariae *et al.* 1998*a*). The phosphatase for CDH1 remains unclear, but Cdc14p, a dual specific phosphatase, seems to be a good candidate, as mutations in Cdc14p cause cells to arrest at late mitosis (Jaspersen *et al.* 1998).

(c) *Substrates of APC*

APC controls multiple events in the cell cycle through degradation of multiple substrates in a precise temporal order (table 1). Among APC substrates, the anaphase inhibitors Pds1p/Cut2p are degraded at the onset of anaphase to trigger sister chromatid separation (Cohen-Fix *et al.* 1996), and the spindle-associated protein Ase1p and mitotic cyclins are degraded at late anaphase to mediate exit from mitosis (Holloway *et al.* 1993; Juang *et al.* 1997). Geminin, an inhibitor of DNA replication, is degraded later in mitosis to allow DNA replication in the succeeding cell cycle (McGarry & Kirschner 1998). CDC20, an activator of APC, is degraded as cells exit from mitosis to allow a switch from CDC20-associated APC to CDH1-associated APC (figure 3*a–c*) (Fang *et al.* 1998*b*; Prinz *et al.* 1998; Shirayama *et al.* 1998). APC also functions in G1 to degrade essential mitotic regulators, such as Plk1 and aurora-related kinase (ARK2), and therefore, to reset the cell cycle (figure *3d*) (Glover *et al.* 1995; Bischoff *et al.* 1998; Charles *et al.* 1998; Fang *et al.* 1998*b*; Shindo *et al.* 1998; Shirayama *et al.* 1998). Thus, APC is a master regulator of the cell cycle, coordinating ordered progression of the cell cycle through late mitosis and G1.

3. INHIBITION OF APC BY THE SPINDLE-ASSEMBLY CHECKPOINT

Overriding the normal progression of the cell cycle are checkpoint controls that respond to defects in the machinery of DNA replication and chromosome segregation. The spindle-assembly checkpoint mechanism ensures equal separation of sister chromatids into two daughter cells by delaying anaphase initiation until all chromosomes are aligned properly at the metaphase plate. APC is not active in the checkpoint-arrested cells and, therefore, is the most likely target for checkpoint intervention. Genetic studies in yeast have identified a group of genes, *MAD1, MAD2, MAD3, BUB1, BUB2, BUB3* and *MPS1,* involved in checkpoint control (Hoyt *et al.* 1991; Li & Murray 1991; Chen *et al.* 1996; Hardwick *et al.* 1996; Li & Benezra 1996). A combination of genetic and biochemical analysis has shown that Mad2p lies most distal from the checkpoint sensory machinery (Elledge 1996; Rudner & Murray 1996). We found that transfection of the human MAD2 gene into HeLa cells causes an arrest of transfected cells at mitosis with condensed chromosomes (figure 4). Similarly, overexpression of Mad2p in yeast also arrests cells at metaphase (He *et al.* 1997). Therefore, overexpression of MAD2 mimics the activation of the spindle-assembly checkpoint (Li *et al.* 1997; Fang *et al.* 1998*a*).

To understand the biochemical mechanism of MAD2-mediated cell cycle arrest, we expressed and purified the recombinant human MAD2 protein and studied its interaction with the cell-cycle machinery. Surprisingly, we found that the recombinant MAD2 protein exists in two folded states: a tetramer and a monomer (Fang *et al.* 1998*a*). These two forms of MAD2 have different biological activity, though they have identical sequence and no measurable post-translational difference as determined by mass spectrometry. When injected into *Xenopus* embryos, the tetramer causes an immediate and stable arrest of injected blastomeres, while injection of the monomer has no effect on cell division. Similarly, while addition of the monomer to mitotic extracts does not affect cyclin degradation, addition of the tetramer inhibits cyclin degradation. This inhibition is due to a direct inhibition of the mitotic APC activity, as APC was not active when purified from mitotic extracts that had been pre-incubated with the MAD2 tetramer. Interestingly, these two forms of MAD2 interconvert only by refolding of the protein upon denaturation. Therefore, the two different forms of MAD2 represent two activity states of the protein and may correspond to two physiological states of MAD2 involved in transducing the checkpoint signal.

MAD2 arrests cells at metaphase by forming a MAD2, CDC20 and APC ternary complex *in vivo*, and thereby inhibiting activation of APC by CDC20 (Fang *et al.* 1998*a*; Hwang *et al.* 1998; Kallio *et al.* 1998; Kim *et al.* 1998; Wassmann & Benezra 1998). Although both the MAD2 tetramer and monomer can bind to CDC20

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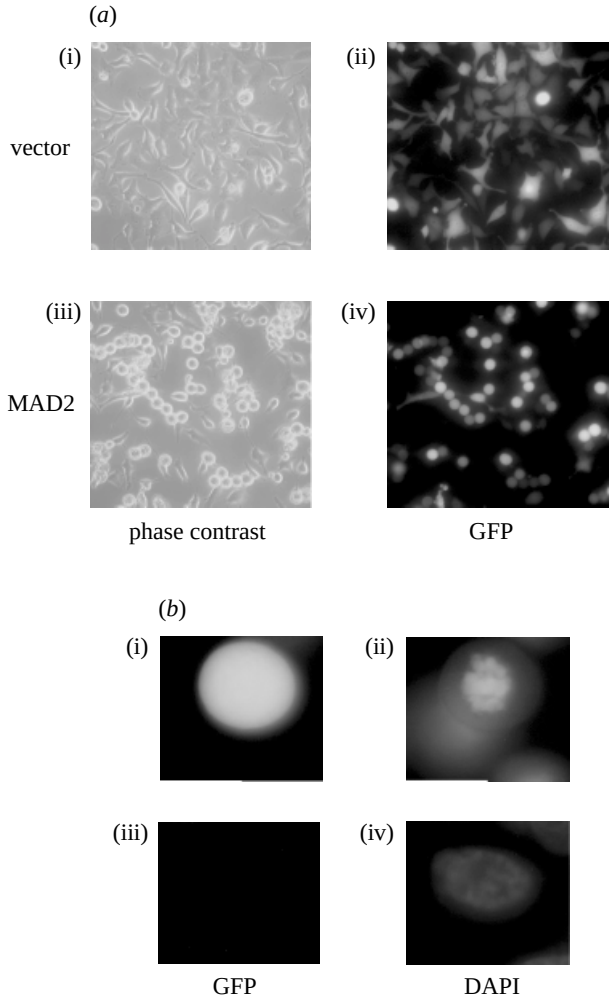

Figure 4. Overexpression of MAD2 arrests HeLa cells in mitosis. (*a*) The human MAD2 gene ((iii) and (iv)) or the control vector ((i) and (ii)) were co-transfected with a small amount of the GFP gene into HeLa cells. Forty-four hours after transfection, cells were imaged under a phase contrast microscope ((i) and (iii)) and for the GFP fluorescence to identify transfected cells ((ii) and (iv)). In the MAD2-transfected dish, there is a drastic increase in the number of mitotic cells (rounded cells). In addition, almost all the GFP-positive cells in the MAD2-transfected dish are in mitosis (iv), while the majority of the GFP-positive cells in the vector-transfected dish are in interphase (ii). (*b*) Two cells from (*a*) (iii) were imaged under high magnification. A MAD2-transfected (GFP-positive; (i) and (ii)) and a MAD2-untransfected (GFP-negative; (iii) and (iv)) cell were imaged for the GFP fluorescence ((i) and (iii)) and for DAPI fluorescence ((ii) and (iv)). The MAD2-transfected cell contains condensed mitotic chromosomes.

in vitro, only the tetramer inhibits APC activation, suggesting that binding itself is not sufficient for inhibition and that a conformation change in MAD2, triggered by checkpoint signals, is required for inhibition of APC (Fang *et al.* 1998*a*). This MAD2–CDC20–APC ternary complex must be readily reversible. When all chromosomes are aligned at the metaphase plate, the checkpoint signal disappears and MAD2 dissociates from the ternary complex. The resulting CDC20–APC complex becomes active in degrading the anaphase inhibitor, leading to separation of sister chromatids (Fang *et al.* 1998*a*).

As unattached kinetochores are present in every mitosis prior to metaphase, the checkpoint mechanism functions in every cell cycle to determine the timing of anaphase initiation. Consistent with this proposal, MAD2 and CDC20 form a complex in every mitosis independent of exogenous activation of the checkpoint (G. Fang and M. W. Kirschner, unpublished data) (Wassmann & Benezra 1998).

4. THE APC CYCLE

A model for the cell-cycle regulation of APC is presented in figure 5 (Fang *et al.* 1998*a*,*b*). As cells enter mitosis, a few subunits of APC are phosphorylated, and phosphorylation of APC increases its affinity to CDC20. The candidate kinases for APC include Plk1 and Cdc2 (Charles *et al.* 1998; Fang *et al.* 1998*b*; Kotani *et al.* 1998; Patra & Dunphy 1998; Shirayama *et al.* 1998). In the presence of unattached kinetochores, MAD2 binds to CDC20 and together they form an inactive ternary complex with APC, which could occur normally in each prometaphase. Presumably the conformational change of MAD2, triggered by the checkpoint pathway, is a highly poised reaction, perhaps in some way autocatalytic, so that the inhibitory MAD2 conformer, shown as rectangles in figure 5, will form the inactive ternary complex. Though the interconversion of active and inactive MAD2 conformers is very slow *in vitro*, it is presumably mediated by protein factors and is much faster *in vivo*. When all the chromosomes are aligned at the metaphase plate, MAD2 re-establishes the original equilibrium and dissociates from APC. The resulting binary CDC20–APC complex triggers the metaphase-to-anaphase transition by degrading the anaphase inhibitor Pds1p.

Throughout early mitosis, CDH1 is unable to associate with APC due to its phosphorylation by the Cdc2 kinase. Later in mitosis, an unidentified mechanism causes dephosphorylation of CDH1. Dephosphorylated CDH1 in turn binds to and activates APC. The association of CDC20 with APC is presumably weak enough for an appreciable off-rate for CDC20 to exist. Alternatively, the same dephosphorylation mechanism for CDH1 may cause changes in APC, which weaken its affinity for CDC20. (The potential complex phosphorylation pattern of the eight APC subunits has not been characterized.) CDC20 then dissociates from APC and the free form of CDC20 is degraded by CDH1-activated APC, leading to a complete replacement of CDH1 for CDC20 and a change of substrate specificity of APC. CDH1 maintains APC activity in G1. The CDH1–APC complex in G1 may maintain the interphase state by inactivating mitotic kinases such as Plk1. In addition, APC in metazoan cells may have special functions in G1 related to cell differentiation and growth control. As cells enter S phase, CDH1 dissociates from APC, again by unknown mechanisms. The APC activity is low in S phase and G2 phase, and in early mitosis until phosphorylation of APC and accumulation of CDC20 restart the cycle.

5. SUMMARY

In summary, APC is a key regulator of the cell cycle and itself is under complex control by a network of positive and negative regulators. The substrates of APC

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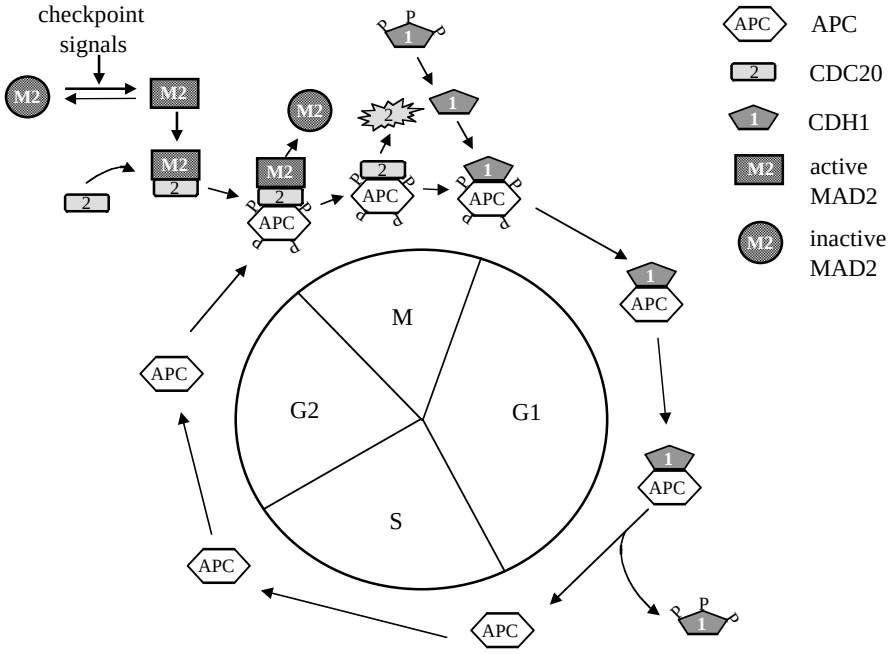

Figure 5. A model for cell-cycle regulation of APC.

include cyclin B and cyclin A, the regulatory subunits of the mitotic Cdc2 kinase. However, in addition to control of mitotic kinase activity, APC is involved in several other important steps of regulation, some of which precede cyclin destruction. The identification of the APC activators, CDC20 and CDH1, allows us to begin to understand how different events during mitosis and in G1 are regulated by the degradation of different substrates in a specific temporal order. We may also find that differential localization of substrate proteins imposes a spatial order on the process. The finding that the spindle-assembly checkpoint pathway acts through inhibiting APC activation, allows us to dissect this checkpoint signalling pathway biochemically. Further studies of the APC regulatory network should lead to a better understanding of the role of proteolysis in the cell-cycle control, but also possibly in other biological regulatory processes.

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Discussion

L. Harrington (*Amgen Research Institute, Ontario, Canada*). Your results suggest that addition of CDH1p might displace CDC20p from the APC. Have you tested this?

G. Fang. This seems unlikely. Both CDC20p and CDH1p are bound to the APC at sub-stoichiometric levels at any stage of mitosis. So it doesn't look like direct competition for binding sites but we haven't tested this directly.

J. Raff (*Wellcome—CRC Institute, Cambridge, UK*). Researchers have recently shown that MAD2 might be required in mammalian cells for normal cell division. Do you have any evidence whether that's the case in your system?

G. Fang. Yes, we have similar data. If we arrest cells at the G1–S boundary and then release them to allow progress into mitosis without activating the spindle checkpoint, MAD2p still associates with CDC20p and the APC. Then at the metaphase-to-anaphase transition, MAD2p dissociates.

H. Yamano (*ICRF Clare Hall Laboratories, Hertfordshire, UK*). You showed APC–CDH1p can ubiquitinate cyclin in a DB-independent manner. Do you know which region of cyclin is involved in recognition?

G. Fang. We are looking at this question at the moment.

A. Hershko (*Technion—Israel Institute for Technology, Haifa, Israel*). You showed CDH1p confers relaxed substrate specificity on the APC/cyclosome. However, *in vivo*, when CDH1p is presumably active, cyclin B is still degraded in a DB-dependent manner. How do you explain this?

G. Fang. Actually, we are puzzled by this. We don't know what CDH1p is recognizing. Once we map the recognition site, we might have a better idea.

A. Hershko. My second question is that I noticed in your paper that the concentrations of MAD2p that you are using are very high, about 10 μM. Why do you need such high concentrations?

G. Fang. We can use much lower concentrations, so these high concentrations are not essential.

R. T. Hunt (*ICRF Clare Hall Laboratories, Hertfordshire, UK*). You've shown nicely that the APC stays active during G1 which is presumably using HCT1p–CDH1p. What turns it off?

G. Fang. In mammalian cells it is presumably cyclin E–cdk2 kinase activity, but this hasn't been shown yet.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES