Regulation of APC Activity by Phosphorylation and Regulatory Factors

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Abstract. Ubiquitin-dependent proteolysis of Cut2/Pds1 and Cyclin B is required for sister chromatid separation and exit from mitosis, respectively. A naphase-promoting complex/cyclosome (APC) specifically ubiquitinates Cut2/Pds1 at metaphase–anaphase transition, and ubiquitinates Cyclin B in late mitosis and G1 phase. However, the exact regulatory mechanism of substrate-specific activation of mammalian APC with the right timing remains to be elucidated. We found that not only the binding of the activators Cdc20 and Cdh1 and the inhibitor Mad2 to A PC, but also the phosphorylation of Cdc20 and Cdh1 by Cdc2-GST-Cyclin B and that of A PC by Polo-like kinase and CA M P-dependent protein kinase, regulate A PC activity. The cooperation of the phosphorylation/dephosphorylation and the regulatory factors in regulation of A PC activity may thus control the precise progression of mitosis.

Key words: anaphase-promoting complex • Cdc20 • Cdh1 • MAPK • phosphorylation

UBQUI TIN-mediated proteolysis plays a critical role in regulation of cell cycle progression, and is a particularly effective method in promoting unidirectional progression in the cell cycle to ensure its irreversibility (Murray et al., 1989; Hershko, 1997; Peters, 1998; Timmermann, 1999; Morgan, 1999). Proteolysis of mitotic cyclin is required for the termination of mitosis. Cyclin B is degraded at the metaphase–anaphase transition and Cdc20 is required for proper mitotic cyclin destruction (Murray et al., 1996; Hershko, 1997; Peters, 1998; Timmermann, 1999; Morgan, 1999). Proteolysis of Cyclin B is important for the progression of metaphase to anaphase transition and exit from mitosis (Murray et al., 1989; Hershko, 1997; Peters, 1998; Timmermann, 1999; Morgan, 1999). Proteolysis of Cyclin B is important for the progression of metaphase to anaphase transition and exit from mitosis (Murray et al., 1989; Hershko, 1997; Peters, 1998; Timmermann, 1999; Morgan, 1999).

A naphase-promoting complex/cyclosome (APC) functions as a cell cycle–regulated ubiquitin ligase that mediates destruction of these cell cycle regulatory factors by the proteasomes during mitosis. It is activated at metaphase–anaphase transition and remains active until late G1 phase (Amon et al., 1994; King et al., 1995; Lahav-Baratz et al., 1995; Sudakin et al., 1995; Iwai et al., 1996; Peters et al., 1996; Yamashita et al., 1996; Hershko, 1997; Peters, 1998; Koepp et al., 1999; Morita et al., 1999). APC is required for the metaphase–anaphase transition and degradation of mitotic cyclin, and its irreversibility (Murray et al., 1989; King et al., 1994, 1995; Ishii et al., 1996; Peters et al., 1996; Yamashita et al., 1996; Hershko, 1997; Peters, 1998; Koepp et al., 1999; Morita et al., 1999). Proteolysis of Cyclin B is important for the progression of metaphase to anaphase transition and exit from mitosis (Murray et al., 1989; Hershko, 1997; Peters, 1998; Timmermann, 1999; Morgan, 1999).

The APC activity has been thought to be regulated by cell cycle–specific phosphorylation and dephosphorylation (Kotani et al., 1995; Lahav-Baratz et al., 1995; Sudakin et al., 1995; Iwai et al., 1996; Peters et al., 1996; Yamashita et al., 1996; Patra and Dunphy, 1998; Yu et al., 1998). It was reported that APC is controlled by Cdc2-Cyclin B (MPF) by reversible phosphorylation (Hershko et al., 1994; Lahav-Baratz et al., 1995; Sudakin et al., 1995; Shteinberg and Hershko, 1999; Shteinberg et al., 1999). Recently, specific kinases that regulate APC activity were identified. Polo-like kinase (Plk) (Glover et al., 1998), a homologue of Drosophila polo and budding yeast Cdc5, phosphorylates at least three A PC subunits, A PC1, A PC3, and A PC6, and activates A PC (Charles et al., 1998; Descombes and Nigg, 1998; Kotani et al., 1998; Shirayama et al., 1998), whereas CA M P-dependent protein kinase (PKA) phosphorylates A PC1 and A PC3 (Kotani et al., 1998) and suppresses A PC activity (Ishii et al., 1996; Yamashita et al., 1996; Yamada et al., 1997; Kotani et al., 1998). Furthermore, PP1 has been found to be required for the activation of A PC in metaphase–anaphase transition (Ishii et al., 1996). Therefore, it became clear that phosphorylation and dephosphorylation of APC plays an important role in regulation of APC activity during mitosis.

On the other hand, recent genetic and biochemical analyses in yeast, Drosophila, and Xenopus have indicated that APC is activated by the WD-repeat proteins, Cdc20/p55CDC/C/fitness (Cdc20) and Cdh1/Hct1/fitness-related (Cdh1), in a substrate-specific manner (Visintin et al., 1997; Schwab et al., 1998; Zachariae et al., 1998a; Yu et al., 1998). The precise mechanism of regulation for APC activity during mitosis remains to be elucidated. We found that not only the binding of the activators Cdc20 and Cdh1 and the inhibitor Mad2 to A PC, but also the phosphorylation of Cdc20 and Cdh1 by Cdc2-GST-Cyclin B and that of A PC by Polo-like kinase and CA M P-dependent protein kinase, regulate A PC activity. The cooperation of the phosphorylation/dephosphorylation and the regulatory factors in regulation of A PC activity may thus control the precise progression of mitosis.
al., 1997; Sigrist and Lehner, 1997; Kallio et al., 1998; Fang et al., 1998a,b; K ramer et al., 1998; L orca et al., 1998; Z acharia et al., 1998b), whereas A PC is inactivated by a spindle assembly checkpoint through Mad2 (H et al., 1997; L i et al., 1997; Fang et al., 1998b; G orbsky et al., 1998; H wang et al., 1998; K allio et al., 1998; K im et al., 1998). Very recently, it was reported that whereas Mad1, Mad2, Mad3/ Bub1, and Bub3 suppress Cdc20-dependent A PC activation, Bub2 localized in the spindle pole body regulates Cdh1-dependent A PC activation (A lexandroiu et al., 1999; F esquet et al., 1999; F raschini et al., 1999). Furthermore, it was found that Cdc14, of which activity is regulated by B ub2/B yr4 and R ENT complex (A lexandroiu et al., 1999; Shou et al., 1999; V isintin et al., 1999), dephosphorylates Cdh1/Hct1 and inactivates A PC (V isintin et al., 1998). Therefore, A PC activation is regulated by at least four distinct mechanisms: activation and inactivation by phosphorylation and dephosphorylation of A PC itself; activation by the binding of substrate-specific activators Cdc20 and Cdh1 to A PC; suppression of A PC activity by the spindle assembly checkpoint through Mad family and Bub family; and regulation of A PC activity by Bub2/RE NT complex system. However, the precise regulatory mechanism of substrate-specific activation of mammalian A PC with the right timing through these complicated mechanisms remains to be elucidated.

We found that not only the binding of the activators Cdc20 and Cdh1 and the inhibitor Mad2 to A PC, but also the phosphorylation of Cdc20 and Cdh1 by M PF and inhibition of A PC by Plk and L K, regulate the timing of A PC activation. We discuss here the regulatory mechanisms of A PC activation.

Materials and Methods

cDNA Cloning and Plasmid Constructs

H uman Cdc20 and M ad2 cDNA constructs were prepared by reverse transcriptase–PCR. Two human expressed sequence tag (EST) EST clones to Cdh1/Hct1 were used as probes for the cloning of full-length human Cdh1 cDNA in a λgt10 human erythroleukemia K 562 cDNA library. The T7- and His-tagged Cdc20, Cdh1, and Mad2 expressed in E. coli were phosphorylated in NovABuf (DE 3) in the presence of 0.5 M MgCl2, 25 mM dithiothreitol, 8 M urea, and 0.5 M MgCl2 at 25°C for 20 h. The cell lysates were solubilized (10 mM Tris, pH 8.0, 0.1 M NaH₂PO₄, and 6 M guanidine isothiocyanate), bound to His-Bind resins, washed (10 mM Tris, pH 8.0, 0.1 M NaH₂PO₄, and 8 M urea) five times, eluted (10 mM Tris, pH 4.5, 0.1 M NaH₂PO₄, and 8 M urea) and dialyzed. Rab t antisera against tagged Cdc20 and Cdh1 were prepared and each specific antibody was purified by antigen affinity chromatography.

In Vitro Kinase Assay

The purified Cdc20, Cdh1, or M ad2 (1 μg) was incubated with 10 μCi of γ-[³²P]AT P (3,000 Ci/mmol) and human Cdc2-GST-Cyclin B prepared by baculovirus (0.1 μg) (K otani et al., 1998) or Plk (0.1 μg) (K otani et al., 1998) in 50 μl of a kinase buffer (M PF: 10 mM Tris, pH 7.4, 10 mM M gCl₂, 0.1 mM E GTA, and 0.05% β-mercaptoethanol; Plk: 20 mM Tris, pH 7.4, 10 mM M gCl₂, 25 mM NaCl, 0.2 mM BSA, and 0.05% β-mercaptoethanol), at 37°C for 30 min. The labeled proteins were immunoprecipitated with anti-T7 ma b (Novagen), washed, and resolved in 7% SDS-PAGE. The ³²P-labeled proteins were immunoprecipitated by anti-Cdc27 antibody and were resolved in 7% SDS-PAGE.

Preparation of APC

M ouse NIH 3T3 cells were maintained in D ME supplemented with 10% F CS. Cells were synchronized at the G1/S boundary by double blocking with 1 μg/ml aphidicolin and harvested 2 h after drug release. A PC in the S phase was prepared by Resource Q chromatography and immunoprecipitation with anti-Cdc27 antibody (a gift of D r. P. H ieter and A. P age, University of British Columbia, Vancouver, Canada) as described (K otani et al., 1998). Immunoprecipitates were washed three times with a buffer containing 500 mM KCl to remove M PF and PIk, and then washed with the buffer for kinase or ubiquitination reaction.

Ubiquitination Assay

Cdc20 and Cdh1 (1 μg) were phosphorylated by incubating with 0.1 μg of Cdc2-GST-Cyclin B and 0.1 mM ATP in 10 μl of M PF kinase buffer at 37°C for 30 min. The phosphorylated Cdc20 and Cdh1 were bound to His-Bind resin, washed with the kinase buffer containing 0.5 M KCl three times to remove M PF, and washed with the ubiquitination buffer. In some cases, the purified A PC was phosphorylated by PKA phosphorylated by M PF (pPIk) and/or PKA in 50 μl of P Ik kinase buffer containing 1 mM ATP, 0.1 μg of human Cdc2-GST-Cyclin B prepared by baculovirus (K otani et al., 1998) and 0.1 μg of PIk, or in PKA buffer (10 mM Tris, pH 7.4, 10 mM M gCl₂, and 0.1 mM EGTA) with 0.5 μg of bovine PKA catalytic subunit, and washed twice with 5 mM Tris, pH 7.6, and 0.5 mM M gCl₂. The phosphorylated or untreated A PC was incubated with 2 μg of GST-Cut2 (a gift of D r. M. Y anagida, Kyoto University, K yoto, J apan) (F unabiki et al., 1997) or 2 μg of human GST-Cdc2-GST-Cyclin B (a gift of D r. N. Watanabe, R IKEN, J apan) in the presence of various amounts (0–1 μg) of GST ubiquitinated or nonubiquitinated forms of Cdc20 and Cdh1 in 20 μl of kinase buffer containing 10 mM M gCl₂, 2 mM ATP, 2 mM DTT, 20 μg of bovine ubiquitin, 0.5 μg of bovine PKA catalytic subunit, and 1 μg of mouse recombinant hE2-C (K otani et al., 1998), and incubated at 35°C for 30 min or the indicated time. The samples were analyzed by 7% SDS-PAGE, and the ubiquitinated forms detected by immunoblotting with anti-T7 antibody.

Results

MPF Phosphorylates Cdc20 and Cdh1

The T7-tagged human Cdc20, Cdh1, and M ad2 were prepared in E. coli, and their possible kinases, human Cdc2-GST-Cyclin B (M PF) prepared by baculovirus and His-tagged PIk produced in E. coli, were homogeneously purified as shown in Fig. 1 A. The in vitro phosphorylation was performed by incubating these recombinant Cdc20, Cdh1, and M ad2 with recombinant M PF or PIk in the presence of γ-[³²P]AT P. Both Cdc20 and Cdh1 could be phosphorylated by M PF (Fig. 1 B, lanes 1 and 3) but not by PIk (Fig. 1 B, lanes 2 and 4) in vitro. M ad2 was not phosphorylated by these kinases (data not shown).

Mpf with but Not without Suc1/Cks1 Phosphorylates APC

Next, we performed in vitro phosphorylation of A PC by...
MPF in the presence or absence of recombinant purified human Suc1/Cks1 (CksHs-1 or CksHs-2). There exist two human Suc1/Cks1 homologues, CksHs-1 and CksHs-2. As shown in Fig. 1 C, none of the APC core subunits could be phosphorylated by MPF alone (Fig. 1 C, lane 1). In the presence of CksHs-1 (Fig. 1 C, lane 3) or CksHs-2 (Fig. 1 C, lane 4), however, MPF could clearly phosphorylate APC1/Tsg24 and APC3/Cdc27, which is consistent with the results of Patra and Dunphy (1998). The MPF-activated Plk could phosphorylate APC1/Tsg24 and APC3/Cdc27 but not APC6/Cdc16, which is consistent with the results of Patra and Dunphy (1998). The MPF-activated Plk could phosphorylate APC1/Tsg24 and APC3/Cdc27, A PC6/Cdc16, and an unidentified protein of 85 kD (Fig. 1 C, lane 2) as we previously showed (Kotani et al., 1998). In the presence of all three components (MPF, CksHs-1 or CksHs-2, and Plk), these APC core subunits were most efficiently phosphorylated (Fig. 1 C, lanes 5 and 6). These results indicate that none of the APC core subunits can be phosphorylated by MPF alone, but in the presence of Suc1/Cks1, two APC core subunits, APC1/Tsg24 and APC3/Cdc27, can be phosphorylated by MPF.

pCdc20 but Not Cdc20 Activates Ubiquitination of Cut2 and Cyclin B

In vitro reconstituted ubiquitination assay was performed to assess the substrate- and time-specific activation or inactivation of mammalian APC, and the effects of phosphorylation of the regulatory factors as well as APC on the ubiquitination activity were determined. Fig. 2, A and B, show that...
the purified APC in the S phase had no activity to ubiquitinate GST-Cut2 (Fig. 2A, lane 1) or GST-Cyclin B (Fig. 2B, lane 1), even in the presence of Cdc20 (Fig. 2A and B, lanes 2 and 3).

Since we found that Cdc20 could be phosphorylated by MPF, we next examined the effect of Cdc20 phosphorylated by MPF (pCdc20) on APC activity. The Cdc20 was phosphorylated by MPF, bound to His-bind resin, and washed with 0.5 M KCl to remove MPF. It was confirmed by the immunoblot and MPF assay that the pCdc20 prepared in this way was free of MPF (data not shown). Interestingly, when the APC was incubated with pCdc20, both GST-Cut2 and GST-Cyclin B were ubiquitinated in a dose-dependent manner (Figs. 2A and B, lanes 4–6). Furthermore, Mad2 inhibited these ubiquitination activities in a dose-dependent manner (Figs. 2A and B, lanes 7 and 8). These results indicated that pCdc20 but not Cdc20 activates ubiquitination of Cut2/Pds1 and Cyclin B, and the pCdc20-dependent APC activation can be suppressed by Mad2.

Cdh1 but Not pCdh1 Activates Ubiquitination of Cyclin B but Not Cut2

No GST-Cut2 was ubiquitinated even in the presence of a large excess of Cdh1 (Fig. 3A, lanes 2 and 3) or Cdh1 phosphorylated by MPF (pCdh1) (Fig. 3A, lanes 4 and 5). Cdh1 was phosphorylated by MPF, bound to His-bind resin, and washed with 0.5 M KCl to remove MPF. Activity to ubiquitinate GST-Cut2 (A) or GST-Cyclin B (B) was measured in the presence of recombinant E1 and hE2-C in the ubiquitination buffer. Reaction mixes were applied to 7% SDS-PAGE, and the polyubiquitinated GST-Cut2 and GST-Cyclin B bands were detected by immunoblotting with anti–Cyclin B antibody and anti–GST antibody, respectively. Polyubiquitinated Cyclin B bands are shown as Ub-Cyclin B. Arrows indicate the positions of molecular mass marker in kilodaltons.
pCdc20, and it contained no MPF activity. The ubiquitination of GST-Cyclin B was activated by Cdh1 in a dose-dependent manner (Fig. 3 B, lanes 1–4), and the activation was not blocked by the addition of a large excess of Mad2 (Fig. 3 B, lane 5). In contrast, even sufficiently large amounts of pCdh1 could not activate the ubiquitination of GST-Cyclin B (Fig. 3 B, lanes 6 and 7). These results indicate that APC in the presence of Cdh1 but not pCdh1, effectively and specifically ubiquitinates Cyclin B (but not Cut2/Pds1) at least in vitro, and that Mad2 suppresses only pCdc20-dependent APC activation.

**Positive and Negative Effects of APC Phosphorylation by pPlk and PKA on Ubiquitination Activity in the Presence of pCdc20 or Cdh1**

Next, we examined the effect of phosphorylation of APC itself on APC activity in the presence of the activated form of the regulatory factors, pCdc20 and Cdh1. As previously reported, the pPlk-activated APC (pAPC) ubiquitinated GST-Cyclin B (Fig. 4, lower panel, lane 1), but its activation was suppressed by phosphorylation with PKA (Fig. 4, lower panel, lane 3). However, pAPC could not ubiquitinate GST-Cut2 (Fig. 4, upper panel, lane 1). Furthermore, if the APC or pAPC was once phosphorylated by PKA, the GST-Cyclin B could not be ubiquitinated even in the presence of the active regulatory factors, pCdc20 or Cdh1 (Fig. 4, lower panel, lanes 4–7). These results indicate that the ubiquitination of Cyclin B is suppressed by PKA as well as by Mad2, and that pPlk specifically stimulates ubiquitination of Cyclin B but not Cut2/Pds1.

**pCdc20 or Cdh1 Acts Synergistically with pPlk on Cyclin B Ubiquitination**

Time course experiments of Cyclin B ubiquitination with pAPC demonstrated that the kinetics of the ubiquitination reaction became much faster in the presence of the active regulatory factors, pCdc20 or Cdh1 (Fig. 5 A and B, lanes 8), i.e., a shorter incubation time (10 min) was enough to reach the saturation level of GST-Cyclin B ubiquitination (Fig. 5, A and B, lane 8), indicating that pCdc20 or Cdh1 acts synergistically with pPlk on Cyclin B ubiquitination. It was also found that Mad2 does not inhibit pAPC activity (Fig. 5 A, lane 10); thus, Mad2 inhibits only pCdc20-dependent APC activation, whereas PKA suppresses both pCdc20- and pPlk-dependent APC activation.

**Binding of Cdc20, pCdc20, and Cdh1 to APC and pAPC**

The binding assay of the regulatory factors to APC demonstrated that both Cdc20 and pCdc20 constitutively bound to APC (Fig. 6 A, lanes 1 and 2) and to pAPC (Fig. 6 A, lanes 7 and 8) but not to APC phosphorylated by PKA (pAPC(PKA)) (Fig. 6 A, lanes 13 and 14). These results indicate that pCdc20-induced APC activation is dependent on the phosphorylation of Cdc20 with MPF rather than on the binding preference of pCdc20 to APC. Furthermore, the binding of Cdc20 and pCdc20 to APC or to pAPC was not affected by the addition of Mad2 (Fig. 6 A, lanes 3, 4, 9, and 10), suggesting that Mad2 inhibition of pCdc20-induced APC activation is not due to the binding inhibition of pCdc20 to APC, but due to the direct functional inhibition of pCdc20 by Mad2. In contrast, Cdh1 could bind to either APC (Fig. 6 A, lane 5) or pAPC (Fig. 6 A, lane 11), whereas pCdh1 did not bind to any forms of APC (Fig. 6 A, lanes 6 and 16). These results suggest that Cdh1-induced APC activation totally depends on the binding of Cdh1 to APC or pAPC. Furthermore, these results suggest that Cdh1 (but not pCdh1) specifically binds to and activates APC, and that the PKA-induced inhibition of APC activity is due to the inhibition of the binding of these active regulatory factors to APC.
Cdc20 but none of the components of the purified APC (Fig. 6 B, lane 1), and that pCdc20 actually bound to APC (Fig. 6 B, lane 1) but pCdh1 could not bind to APC (Fig. 6 B, lane 2). These results also confirmed that there is no need to consider the effect of phosphorylation of APC by MPF on ubiquitination activity.

**MPF Phosphorylates Cdc20 and Cdh1 during Mitosis In Vivo**

To demonstrate that MPF phosphorylates Cdc20 and Cdh1 during mitosis in vivo, the immunoprecipitates of anti–Cyclin B-specific antibody from mitotic K 562 cell extracts were incubated with γ[^32P]ATP in MPF kinase buffer. The sample was washed with 1% SDS, diluted 25-fold with the kinase buffer, and the labeled Cdc20 and Cdh1 were immunoprecipitated with anti–Cdc20- or anti–Cdh1-specific antibody, respectively. The[^32P]-labeled Cdc20 and Cdh1 can be clearly seen (Fig. 7, lanes 2 and 3). Fig. 7, lane 1, shows the total[^32P]-labeled proteins without immunoprecipitation by anti–Cdc20 or anti–Cdh1 antibody, and the most prominent band corresponded to the[^32P]-labeled Cyclin B as shown. The preimmune antibody could immunoprecipitate none of these proteins (Fig. 7, lane 4). The phosphorylation could not be observed in cell extracts prepared from cells in S phase (data not shown). These results strongly suggest that MPF phosphorylates Cdc20 and Cdh1 during mitosis in vivo, while it cannot be completely ruled out the possibility that another kinase coimmunoprecipitated with anti–Cyclin B antibody phosphorylates these proteins.

**Binds to APC during Mitosis**

Next, we examined in vivo phosphorylation of Cdc20 and Cdh1 during mitosis and the interaction of these phosphorylated factors with APC in vivo. K 562 cells were labeled with[^32P]orthophosphate during mitosis in vivo and the APC was purified by immunoprecipitation with anti–Cdc27 antibody. The[^32P]-labeled Cdc20 could be immunoprecipitated with anti–Cdc20-specific antibody from this purified APC (Fig. 8, lane 1) and from the total cell lysates (Fig. 8, lane 2), indicating that Cdc20 can indeed be phosphorylated and binds to APC during mitosis in vivo. In contrast, the[^32P]-labeled Cdh1 could not be immunoprecipitated with its specific antibody (Fig. 8, lane 3) from the purified APC, whereas the[^32P]-labeled Cdh1 could be immunoprecipitated from the total cell lysates (Fig. 8, lane 4), demonstrating that Cdh1 can actually be phosphorylated but pCdh1 cannot interact with APC during mitosis in vivo. These results perfectly agree with the findings shown in the in vitro reconstituted system (Figs. 1–6).

**Discussion**

Taken together with the findings described above and other observations recently reported, the scheme of regulation of APC activity is depicted in Fig. 9. We showed in this paper that phosphorylation of Cdc20 is required for Cdc20-dependent APC activation at least in vitro (Fig. 2). It has been reported that Cdc20 is expressed during G2 phase and mitosis (Fang et al., 1998a; Kramer et al., 1998) and binds to APC (Figs. 6 A and 8) in early mitotic stages.
(Fang et al., 1998b). Whereas, Fang et al. (1998a) described that the in vitro translated Cdc20, which might be phosphorylated during preparation, activated APC in vitro and that the phosphatase treatment of Cdc20 had no effect on APC activity, it was recently demonstrated that Cdc20 is clearly phosphorylated during mitosis in HeLa cells (Kramer et al., 1998). Actually, the possible Cdc2 phosphorylation site is conserved in budding yeast Cdc20, fission yeast Slp1, and mammalian Cdc20. Therefore, it is most likely that Cdc20 is indeed phosphorylated during mitosis in vivo. Taken together, we concluded that Cdc20 activates APC when it is phosphorylated by MPF.

The activation of APC can be blocked by the binding of Mad2 to pCdc20 (Fig. 2, A and B; He et al., 1997; Li et al., 1997; Fang et al., 1998; Gorbsky et al., 1998; Hwang et al., 1998; Kallio et al., 1998; Kim et al., 1998). Therefore, at least two events may be required to activate APC at metaphase–anaphase transition: release of Mad2 from pCdc20 after spindle assembly checkpoint is released, and phosphorylation of Cdc20 bound to APC by MPF or binding of pCdc20 to APC. Furthermore, we found that at least in vitro APC activation can be suppressed by PKA (Figs. 4 and 6 A; Ishii et al., 1996), which phosphorylates two APC subunits, APC1 and APC3 (Kotani et al., 1998). Thus, dephosphorylation of PKA phosphorylation sites on APC by a specific phosphatase yet unidentified, which may be PP1 as previously suggested (Ishii et al., 1996), might also be required for the onset of anaphase.

M ad2 cannot inhibit Cdh1-induced APC activation (Fig. 3 B), and pAPC can ubiquitinate Cyclin B even in the presence of Mad2 (Fig. 5 A). Thus M ad2 inhibits APC activation only through Cdc20. Further, the binding of Cdc20 or pCdc20 to APC or pAPC was not affected by M ad2 (Fig. 6 A), indicating that M ad2 inhibits the function of pCdc20 but not the binding of pCdc20 to APC. Very recently, it was reported that whereas M ad1, M ad2, M ad3/ Bub1, and Bub3 suppress Cdc20-dependent APC activation, Bub2 localized in the spindle pole body regulates Cdh1-dependent APC activation (Alexandru et al., 1999; Fesquet et al., 1999; Fraschini et al., 1999). Further detailed functional analyses of the M ad family and Bub family are required to clarify the molecular mechanisms of spindle assembly checkpoint and of APC regulation.
It was clearly shown that pAPC can ubiquitinate Cyclin B but not Cut2 in vitro, but it remains unclear whether free pAPC actually exists during mitosis in vivo. It is most likely that the majority of pAPC forms a complex with either pCdc20 or Cdh1 that ubiquitimates Cyclin B in vivo. Furthermore, phosphorylation of APC by pPlk may not be essential for APC activation at the metaphase–anaphase transition, but our results suggest that it is required for complete Cyclin B ubiquitination in later stages of mitosis.

We showed here that pCdc20 and Cdh1, but neither Cdc20 nor pCdh1 activates APC in vitro, and the following switching mechanism from pCdc20 to Cdh1 could be specified. When the cells enter anaphase after Cut2/Pds1 is entirely degraded, the pAPC–pCdc20 complex steadily ubiquitimates Cyclin B and, consequently, MPF activity decreases by the end of mitosis. The pCdc20 can be dephosphorylated by a specific phosphatase and released from pAPC or it may be degraded as previously suggested (Fang et al., 1998a; Shirayama et al., 1998). When MPF activity is high, Cdh1 is phosphorylated and remains inactive, but when Cdc14 is activated, Cdh1 is dephosphorylated by Cdc14 as suggested (Visintin et al., 1998, 1999; Shou et al., 1999) and binds to and activates APC. Consequently, the pAPC–pCdc20 complex is replaced by the pAPC–Cdh1 (or APCCdh1) complex in late mitosis. The pAPC–Cdh1 complex further ubiquitimates Cyclin B in late mitosis and G1 phase. Thus, the switch mechanism from pCdc20 to Cdh1 may be dependent upon the MPF activity during mitosis, and the MPF activity itself is controlled by the pCdc20- and Cdh1-dependent APC activity.

Cdc20 has been reported to activate ubiquitination of the factors regulating sister chromatid separation and Cdh1 promotes ubiquitination of mitotic cyclins (Visintin et al., 1997; Shirayama et al., 1998). However, it was recently found that in early embryos, Cdh1 is not expressed (Lorca et al., 1998), which is consistent with the observation that Cdh1 is not expressed before stage 13 in Drosophila embryos (Sigrist and Lehner, 1997). It was also showed that pCdc20 regulates ubiquitination of both Cut2/Pds1 and Cyclin B in the early embryonic cell cycle (Lorca et al., 1998). Very recently, Clute and Pines (1999) have demonstrated that Cdc20-dependent proteolysis of Cyclin B begins at the metaphase–anaphase transition in HeLa cells, which is consistent with the results obtained with clam embryo (Clute et al., 1992). These findings are consistent with the observation that pCdc20 can activate ubiquitination not only of Cut2/Pds1, but also of Cyclin B. Thus, pCdc20 alone may be enough for cells to go through mitosis without Cdh1. However, in the somatic cell cycle, Cdh1 in addition to pCdc20 may be required for effective and complete ubiquitination of Cyclin B in later stages of mitosis and G1 phase.

We demonstrated in this paper that Cdh1 can be phosphorylated by MPF (Figs. 1 B and 7), and Cdh1 but not pCdh1 binds to and activates APC only after dephosphorylation (Figs. 3 B, 6, A and B, and 8). These in vitro results

![Figure 8](image-url) Interaction of pCdc20 and Cdh1 with APC during mitosis in vivo. K562 cells were labeled with [32P]-orthophosphate during mitosis, and the APC was purified by Resource Q chromatography and immunoprecipitation with anti-Cdc27 antibody. The [32P]-labeled Cdc20 was immunoprecipitated with anti-Cdc20 antibody from this purified APC (lane 1) and from the total cell lysates (lane 2). The [32P]-labeled Cdh1 was immunoprecipitated with its specific antibody from the purified APC (lane 3) and from the total cell lysates (lane 4). Arrows indicate pCdc20 and pCdh1 phosphorylated in vivo.

![Figure 9](image-url) Model of regulation of APC activity.
are consistent with in vivo data in budding yeast that the dephosphorylated form of Cdk1/Hct1 activates APC (Zachariae et al., 1998b). It was recently described that Cdh1 is constantly expressed throughout the cell cycle (Fang et al., 1998a; Ramesh et al., 1998), binds to APC in late mitosis and G1 phase (Fang et al., 1998b), and is phosphorylated during mitosis in HeLa cells (Kramer et al., 1998). It was also reported that Cdh1/Hct1 binding to APC is regulated by cyclin-dependent kinases (Zachariae et al., 1998b). Very recently, it was demonstrated that Cdc14 dephosphorylates Cdh1/Hct1 and inactivates APC (Visintin et al., 1999). Further, it was found that the activation of Cdc14 is regulated by Bub2/Byr4 and RENT complex (Alexander et al., 1999; Shou et al., 1999; Visintin et al., 1999), although it remains unresolved whether the regulation by RENT complex works in the mammalian system. All these results in vivo agree well with our results in vitro.

In our scheme, it is possible that Cdh1–APC complex activity is maintained until late G1 phase, while it might be diminished by the phosphorylation of Cdh1 with Cdk2, Cdk4, or another unidentified specific kinase that is active at G1/S transition (Amon et al., 1994). It has been reported that the low level of Cyclin B is translated even in G1 phase (Brandes and Hunt, 1996). In the G1 phase, APC–Cdh1 complex may, thus, effectively ubiquitinate this newly translated Cyclin B to avoid activation of MPF. The possible involvement of G1/S Cdns or other specific kinases in Cdh1-dependent APC inactivation must be further studied.

Patra and Dunphy (1998) recently reported that Cdc14 in Xenopus phosphorylates APCR3/Cdc27 and APC6/Cdc16, whereas none of the core APC subunits could be phosphorylated by activated Plk. We confirmed that in the presence of human Suc1/Cks1 (Cks1S1 or Cks1L), Xenopus APC phosphorylates APCR1/Tsg24 and APC6/Cdc16, whereas none of the core APC subunits could be phosphorylated by activated Plk. In our scheme, it is possible that Cdh1–APC complex activity is maintained until late G1 phase, while it might be diminished by the phosphorylation of Cdh1 with Cdk2, Cdk4, or another unidentified specific kinase that is active at G1/S transition (Amon et al., 1994). It has been reported that the low level of Cyclin B is translated even in G1 phase (Brandes and Hunt, 1996). In the G1 phase, APC–Cdh1 complex may, thus, effectively ubiquitinate this newly translated Cyclin B to avoid activation of MPF. The possible involvement of G1/S Cdns or other specific kinases in Cdh1-dependent APC inactivation must be further studied.

Our findings in vitro and in vivo are consistent with the other in vivo observations (Fang et al., 1998a; Ramesh et al., 1998; Lorca et al., 1998), and strongly support the notion that pCdc20 but not Cdc20 activates APC, and that Cdh1 but not pCdk1 binds to and activates APC. Therefore, phosphorylation and dephosphorylation of APC regulatory factors by MPF are critical for their binding to APC and/or APC activation. The MPF activity itself is regulated by the pCdc20- and Cdk1-dependent APC activity, and this feedback control precisely regulates APC activity.

Taken together, the APC activity is regulated by the phosphorylation and dephosphorylation of APC and of the regulatory factors, Cdc20 and Cdh1, by MPF, Plk, PKA, and PP1, as well as by the binding of positive and negative regulatory factors, Cdc20, Cdh1, and Mad2, to APC. These elaborate regulatory mechanisms might control the precise progression of mitosis.

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Drs. Tanaka, Yasuda, and Todokoro retract the above referenced manuscript. An investigation by RIKEN determined that some of the experimental data had been falsified. Details of the investigation are available at http://www.riken.jp/r-world/info/release/press/2004/041224/index_e.html.