

Regulation of the Anaphase-promoting Complex by the Dual Specificity Phosphatase Human Cdc14a*

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Two forms of the anaphase-promoting complex (APC) mediate the degradation of critical cell cycle regulators. APC^{Cdc20} promotes sister-chromatid separation by ubiquitinating securin, whereas APC^{Cdh1} ubiquitinates mitotic cyclins, allowing the exit from mitosis. Here we show that phosphorylation of human Cdh1 (hCdh1) by cyclin B-Cdc2 alters the conformation of hCdh1 and prevents it from activating APC. A human homologue of yeast Cdc14, human Cdc14a (hCdc14a), dephosphorylates hCdh1 and activates APC^{Cdh1}. In contrast, hCdc14a does not affect the activity of APC^{Cdc20}. hCdc14a is a major phosphatase for hCdh1 and localizes to centrosomes in HeLa cells. Therefore, hCdc14a may promote the activation of APC^{Cdh1} and exit from mitosis in mammalian cells.

Orderly progression through the cell cycle relies on the activity of a large ubiquitin protein ligase, the anaphase-promoting complex (APC)¹ or cyclosome (1–4). APC requires two related WD40 repeat-containing cofactors, Cdc20 and Cdh1, for the recruitment and selection of various substrates at different stages of the cell cycle (5–9). During the metaphase-to-anaphase transition, APC^{Cdc20} mediates the ubiquitination and degradation of the securin protein, leading to the activation of the separase, dissolution of the cohesin complex, and chromatid separation (3). In late anaphase, APC^{Cdh1} is turned on and ubiquitinates cyclin B, thus inactivating the cyclin B-Cdc2 mitotic kinase and triggering the exit from mitosis (2–4, 10). The substrates of APC^{Cdc20} contain the destruction box (D box) motif, whereas APC^{Cdh1} has a broader substrate specificity (8). In addition to ubiquitinating D box-containing substrates, APC^{Cdh1} promotes the ubiquitination of additional mitotic regulatory proteins containing the KEN box motif, thus resetting the cell cycle (11). To ensure that the APC^{Cdh1} substrates are degraded after chromatid separation, APC^{Cdh1} has to be activated at a later time as compared with APC^{Cdc20} (10). Therefore, the conversion from the APC^{Cdc20} complex to APC^{Cdh1} is tightly regulated during mitosis (4, 10).

The mechanism of the activation of APC^{Cdh1} and exit from

mitosis is best characterized in budding yeast (4). Mutations of a set of yeast genes, including *TEM1*, *LTE1*, *DBF2*, *DBF20*, *CDC5*, *CDC14*, *CDC15*, and *MOB1*, stabilize mitotic cyclin Clb2p and cause late anaphase arrest, indicating that they are required for the exit from mitosis (4, 12–14). These genes are collectively referred to as the mitotic exit network (MEN) (13). Among the MEN genes, Tem1 is a small GTP-binding protein that belongs to the Rab subfamily (4, 15). Lte1 is a guanine nucleotide exchange factor for Tem1 and presumably regulates its activity positively (4, 16). Lte1 and Tem1 remain spatially segregated until late anaphase, at which time Lte1 and Tem1 co-localize to the bud (17). The activation of Tem1 then triggers the dissociation of the Cdc14 dual specificity protein phosphatase from the RENT (for regulator of nucleolar silencing and telophase) complex, resulting in the release of Cdc14 from the nucleolus (13, 14, 18). In early mitosis, the yeast Cdh1 is phosphorylated by the Clb2-Cdc28 mitotic kinase, and phosphorylation of Cdh1 prevents its interaction with APC (19). In late anaphase, the Cdc14 protein liberated from the nucleolus dephosphorylates Cdh1 and activates APC^{Cdh1} (20). Therefore, Cdc14 may be the most downstream component of the MEN proteins.

Homologues of several yeast MEN proteins, such as Cdc14, Cdc5, and Mob1 have been identified in vertebrates (21–23). In particular, the polo-like kinases, the vertebrate homologs of Cdc5, have been implicated in the activation and maintenance of APC activity (24). Moreover, phosphorylation of human Cdh1 (hCdh1) blocks its ability to activate APC (25, 26). These findings suggest that similar mechanisms for the regulation of APC^{Cdh1} and the exit from mitosis may exist in mammalian cells. However, little is known about whether and how the MEN genes might regulate APC^{Cdh1} in organisms other than the budding yeast.

Here we report the biochemical characterization of a human homologue of Cdc14, hCdc14a. Phosphorylation of hCdh1 by cyclin B1-Cdc2 alters the conformation of hCdh1 and blocks its ability to stimulate the ligase activity of APC. The purified hCdc14a protein dephosphorylates hCdh1 and restores its ability to activate APC. Expectedly, hCdc14a does not affect the activity of APC^{Cdc20}. Moreover, hCdc14a exists as part of a 500-kDa complex and is a major phosphatase of hCdh1 in HeLa cells. Finally, hCdc14a localizes to centrosomes throughout the cell cycle. Because several yeast MEN proteins have been shown to localize to spindle pole bodies, the centrosome localization of hCdc14a is consistent with it playing an important role in the regulation of APC in mammalian cells. Therefore, our data suggest that hCdc14a activates APC^{Cdh1} in late anaphase and is a member of the MEN family of proteins in mammals.

EXPERIMENTAL PROCEDURES

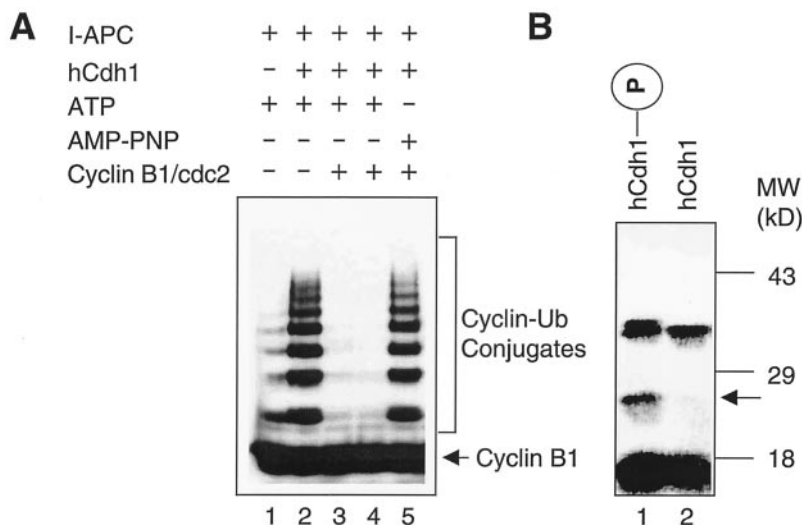
Protein Expression and Purification—For the production of hCdc14, the phosphatase-inactivating mutant of hCdc14 with its active site

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¹ The abbreviations used are: APC, anaphase-promoting complex; hCdh1, hCdc14a, and hCdc20, human Cdh1, Cdc14a, and Cdc20, respectively; MEN, mitotic exit network; GST, glutathione S-transferase; AMP-PNP, adenosine 5'-(β , γ -imino)triphosphate.

FIG. 1. Human cyclin B1-Cdc2 inactivates APC^{Cdh1} in an ATP-dependent manner. **A**, interphase APC (*I*-APC) from interphase *Xenopus* egg extracts exhibited basal level ubiquitination activity (*lane 1*). The addition of hCdh1 greatly stimulated the ligase activity of APC (*lane 2*). Treatment of hCdh1 with cyclin B1-Cdc2 inhibited its ability to activate APC (*lanes 3 and 4*). The reaction in *lane 4* contained twice as much cyclin B1-Cdc2 as that of *lane 3*. In the presence of AMP-PNP, cyclin B1-Cdc2 failed to inhibit APC^{Cdh1} (*lane 5*). **B**, *in vitro* translated ³⁵S-labeled hCdh1 protein was treated in the presence (*lane 1*) or absence (*lane 2*) of cyclin B1-Cdc2. Chymotrypsin was added to the reaction mixture at a final concentration of 1 μ g/ml for 10 min. The reactions were quenched with SDS sample buffer and analyzed by SDS-PAGE followed by autoradiography. The extra 25-kDa proteolytic fragment of phosphorylated hCdh1 was labeled.



cysteine mutated to a serine (hCdc14-PD), hCdc20, and hCdh1 proteins, recombinant baculoviruses encoding these proteins fused at the N-termini with the His₆ tag were constructed using the Bac-to-Bac system (Life Technologies, Inc.). Sf9 cells were infected with the appropriate viruses for 50 h and lysed with a buffer containing 20 mM Tris (pH 7.7), 150 mM NaCl, and 0.1% Triton X-100. The proteins were incubated with Ni²⁺-NTA beads (Qiagen) and eluted with a step gradient of imidazole. For the production of phosphorylated hCdh1 protein, the Sf9 cells were treated with 0.5 μ M of okadaic acid for 4 h prior to harvesting.

For the production of active cyclin B1-Cdc2 kinase, we constructed baculoviruses encoding human cyclin B1 and an AF mutant of human Cdc2. The Cdc2-AF mutant changes Thr¹⁴ and Tyr¹⁵ of human Cdc2 to alanine (A) and phenylalanine (F), respectively, thereby eliminating the potential inhibitory phosphorylation at these two sites by Wee1 and Myt1. The recombinant cyclin B1-Cdc2 complex was then purified through the glutathione *S*-transferase moiety on Cdc2 after co-infecting Sf9 cells with the cyclin B and Cdc2 baculoviruses.

Kinase and Phosphatase Assays—For the kinase assay, cyclin B1-Cdc2 was incubated with hCdh1N and [γ -³²P]ATP in the kinase buffer (20 mM HEPES, pH 7.7, 50 mM NaCl, 10 mM MgCl₂, 2 mM EDTA, and 0.02% Triton X-100). Reactions were incubated for 1 h at room temperature and were either quenched with SDS sample buffer or used for subsequent phosphatase assays. For the phosphatase assay, purified His₆-hCdc14a or hCdc14a-containing column fractions of HeLa cell lysates were added to the kinase reaction mixture and incubated for 1 h and then quenched with SDS sample buffer. The samples were then separated on SDS-PAGE followed by autoradiography.

APC Ubiquitination Assay—For the purification of interphase APC, the α -APC3 (Cdc27) beads were incubated with 10 volumes of interphase *Xenopus* egg extracts for 2 h at 4 °C and washed five times with XB containing 500 mM KCl and 0.5% Nonidet P-40 and twice with XB. The interphase APC beads were then incubated for 1 h at room temperature with hCdc20 or hCdh1 proteins in the presence or absence of cyclin B1-Cdc2 or hCdc14a. After incubation, the APC beads were washed twice with XB and assayed for cyclin ubiquitination activity. Each ubiquitination assay was performed in a volume of 5 μ l. The reaction mixture contained an energy-regenerating system, 150 μ M bovine ubiquitin, a 5 μ M concentration of the Myc-tagged N-terminal fragment of human cyclin B1, 5 μ M human E1, 2 μ M UbcH10, and 2 μ l of the APC beads. The reactions were incubated at room temperature for 1 h, quenched with SDS sample buffer, and analyzed by SDS-PAGE followed by immunoblotting with α -Myc.

Cell Culture—HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in the presence of 2 mM thymidine (Sigma) for 18 h to arrest the cell cycle at the G₁/S boundary, washed with phosphate-buffered saline, and grown in fresh medium without thymidine for 8 h. Cells were then incubated with 2 mM thymidine for another 18 h, transferred into fresh medium, and harvested at various time points. The cells were lysed with the Nonidet P-40 lysis buffer. HeLa lysates were then fractionated on a Superose 6 column and blotted with α -hCdc14a.

Immunofluorescence—The α -hCdc14a antibody was made against a glutathione *S*-transferase fusion protein containing a C-terminal fragment (residues 330–580) of hCdc14a. The fusion protein was expressed

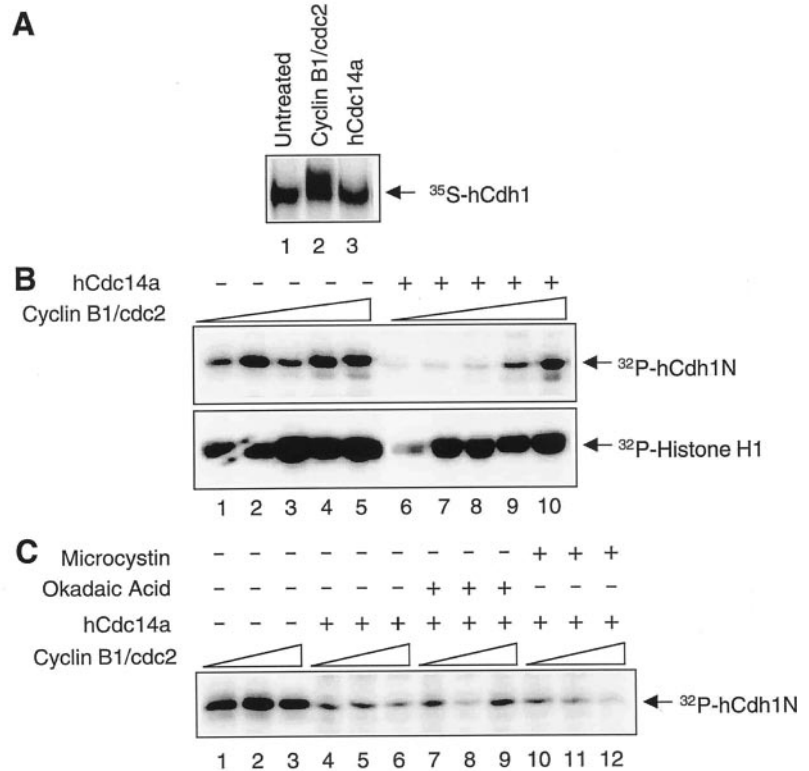
in bacteria and purified with glutathione-agarose beads and used to immunize rabbits at Zymed Laboratories Inc. For immunostaining, HeLa cells grown to 50–70% confluency were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline, and incubated with 1 μ g/ml affinity-purified polyclonal α -hCdc14a and a 1:2000 dilution of α -tubulin monoclonal antibody. After washing, fluorescent secondary antibodies against rabbit and mouse IgG were added at a 1:500 dilution. The cells were again washed three times in phosphate-buffered saline and viewed using a \times 63 objective on a Bio-Rad confocal microscope.

RESULTS

Phosphorylation of hCdh1 by Cyclin B1-Cdc2 Blocks Its Ability to Activate APC—Because Clb2-Cdc28 inactivates Cdh1 during mitosis in yeast (19), we expected that cyclin B1-Cdc2 might act similarly in vertebrates. We therefore tested whether purified cyclin B1-Cdc2 inhibited the activity of APC^{Cdh1} in ubiquitination assays. As shown in Fig. 1A, incubation of the interphase APC with purified hCdh1 protein produced in Sf9 cells greatly stimulated its ubiquitination activity. The addition of cyclin B1-Cdc2 in the presence of ATP significantly reduced the ability of hCdh1 to activate APC (Fig. 1A, lanes 3 and 4). Because the cyclin B1 subunit of the recombinant cyclin B1-Cdc2 kinase is a substrate of APC, it is conceivable that cyclin B-Cdc2 might inhibit APC through substrate competition rather than phosphorylation of Cdh1. To rule out this possibility, we performed the same assay in the presence of an ATP analogue, AMP-PNP. Because the ubiquitin-activating enzyme hydrolyzes the α - β bond of ATP (27), AMP-PNP is fully capable of supporting the ubiquitination reaction. However, AMP-PNP cannot support the phosphorylation reaction as kinases transfer the γ -phosphate to their protein substrates. As expected, incubation of cyclin B1-Cdc2 with hCdh1 in the presence of AMP-PNP did not inactivate APC^{Cdh1}, indicating that the inhibition of hCdh1 by cyclin B1-Cdc2 was not due to substrate competition.

It has been previously shown that phosphorylated hCdh1 has a decreased affinity toward APC (25, 26), which could be caused by electrostatic repulsion between APC and the additional phosphate groups on hCdh1. Alternatively, phosphorylation might also cause a conformational change of hCdh1, preventing it from binding to APC. To test this hypothesis, we performed limited protease digestion of hCdh1 phosphorylated by cyclin B1-Cdc2 and the unphosphorylated hCdh1 (Fig. 1B). Limited protease digestion of the unphosphorylated hCdh1 protein by chymotrypsin yielded two major proteolytic products of 16 and 35 kDa, which presumably corresponded to the N-terminal domain and the C-terminal WD40 domain of hCdh1,

FIG. 2. hCdh1 is a substrate of hCdc14a. **A**, phosphorylation of hCdh1 by cyclin B1-Cdc2 resulted in the appearance of slower migrating hCdh1 species (compare lanes 1 and 2), which was removed by the addition of hCdc14a (lane 3). **B**, an N-terminal fragment of hCdh1 (hCdh1N) (lanes 1–5, top panel) and histone H1 (lanes 1–5, bottom panel) were efficiently phosphorylated by cyclin B1-Cdc2, as revealed by [32 P]PO $_4$ incorporation. Incubation of the kinase reaction mixture with hCdc14a led to efficient dephosphorylation of hCdh1N (lanes 6–10, top panel) but not histone H1 (lanes 6–10, bottom panel). **C**, hCdh1N was phosphorylated by cyclin B1-Cdc2 (lanes 1–3). hCdc14a efficiently dephosphorylated hCdh1N (lanes 4–6). The phosphatase activity of hCdc14a toward hCdh1N was not inhibited by okadaic acid (lanes 7–9) or microcystin (lanes 10–12).



respectively (Fig. 1B). In addition to the two major proteolytic fragments, digestion of the phosphorylated hCdh1 protein with chymotrypsin resulted in the appearance of an additional band at 25 kDa (Fig. 1B). This indicated that phosphorylation indeed induced a conformational change in hCdh1.

hCdc14a Is a Functional Phosphatase for hCdh1—Li *et al.* identified a human homologue of the yeast Cdc14, hCdc14a, on the basis of sequence similarity (21). The hCdc14a protein is 64% identical to yeast Cdc14 (21). Furthermore, overexpression of hCdc14 rescued the temperature-sensitive phenotype of *cdc14-1^{ts}* in budding yeast (21). These data suggest that hCdc14a might be functionally related to yeast Cdc14. To determine whether hCdc14a is a functional phosphatase for hCdh1, purified recombinant hCdc14a protein was added to kinase assays containing cyclin B1-Cdc2 and 35 S-labeled hCdh1. Incubation of hCdh1 with cyclin B1-Cdc2 generated slower migrating phosphorylated species of hCdh1 (Fig. 2A). The addition of hCdc14a reversed the mobility shift of hCdh1, indicating that hCdc14a dephosphorylated hCdh1 (Fig. 2A).

We also performed kinase and phosphatase assays in the presence of [γ - 32 P]ATP (Fig. 2B). Cyclin B1-Cdc2 phosphorylated both histone H1 and an N-terminal fragment of hCdh1 (hCdh1N) (Fig. 2B, lanes 1–5). The inclusion of hCdc14a in the kinase assays greatly reduced hCdh1N phosphorylation, while it dephosphorylated histone H1 to a much lesser extent (Fig. 2B, lanes 6–10). Therefore, hCdh1 is a substrate of the hCdc14a phosphatase. Because recombinant hCdc14a was purified from Sf9 cells, it is possible that there was a trace amount of other phosphatase contamination in our preparation of hCdc14a. To rule out this possibility, high concentrations of microcystin or okadaic acid were added to the hCdc14a reactions. The two most abundant phosphatases in cells are PP1 and PP2A, both of which can be inhibited by microcystin or okadaic acid. In contrast, hCdc14a belongs to the family of dual specificity phosphatases, which use distinct mechanisms for catalysis. As expected, the phosphatase activity of hCdc14a was not inhibited by either microcystin or okadaic acid (Fig. 2C).

hCdc14a Activates APC^{Cdh1}—We next tested whether dephosphorylation of hCdh1 by hCdc14a restored the ability of hCdh1 to activate APC. To obtain phosphorylated hCdh1 protein in large quantities, we treated Sf9 cells infected by hCdh1-encoding baculoviruses with okadaic acid, which inhibited abundant phosphatases and promoted a mitotic-like state in these cells (25). The hCdh1 protein purified from okadaic acid treated cells was hyperphosphorylated. Consistent with an earlier report (25), phosphorylated hCdh1 protein was much less active in stimulating the APC activity, as compared with hCdh1 purified from untreated Sf9 cells (Fig. 3A). The addition of hCdc14a dramatically increased the ability of phosphorylated hCdh1 to stimulate the ligase activity of APC (Fig. 3B). Interestingly, hCdc14a also slightly enhanced the activity of hCdh1 purified from Sf9 cells not treated with okadaic acid (Fig. 3B). It is possible that a portion of Cdh1 protein purified from Sf9 cells was phosphorylated even in the absence of okadaic acid treatment. To estimate the activity enhancement of APC^{Cdh1} by hCdc14a, we repeated the experiment with much less hCdh1 added to the reactions (Fig. 3C). The activity of hCdc14a-treated APC^{Cdh1} at 20 nM was similar to that of APC activated with 400 nM of phosphorylated hCdh1. Therefore, hCdc14a enhanced the activity of phosphorylated APC^{Cdh1} by about 20-fold. To determine whether the phosphatase activity of hCdc14a was required for this stimulation, we constructed, expressed, and purified a phosphatase-inactive mutant of hCdc14a by replacing its active site cysteine with serine. The addition of the phosphatase-dead mutant of hCdc14a (hCdc14a-PD) only stimulated the activity of APC^{Cdh1} slightly (Fig. 3D). Therefore, the phosphatase activity of hCdc14a is required for its ability to activate APC^{Cdh1}.

To ascertain that the stimulatory effect of hCdc14a was specific to Cdh1, we tested the effect of hCdc14a addition on the activity of the APC^{Cdc20} complex. Expectedly, hCdc14a did not affect the activity of APC^{Cdc20} (Fig. 4). Therefore, dephosphorylation of hCdh1 by hCdc14a was probably responsible for the observed stimulatory effect of hCdc14a.

FIG. 3. hCdc14a stimulates the activity of APC^{Cdh1}. *A*, the addition of unphosphorylated hCdh1 protein stimulated the ligase activity of *Xenopus* interphase APC (*I-APC*) (compare lane 1 and lanes 2–6). Phosphorylation of hCdh1 greatly reduced its ability to stimulate the APC activity (lanes 7–11). *B*, same as *A*, except that hCdc14a was included in lanes 2–11. *C*, same as *B*, except that less hCdh1 protein was used in the reactions. *D*, stimulation of APC^{Cdh1} depends on the phosphatase activity of hCdc14a. The hCdc14a mutant protein that does not possess phosphatase activity (hCdc14a-PD) was much less efficient in stimulating APC^{Cdh1} (lanes 10–13), as compared with the wild-type hCdc14a (lanes 6–9).

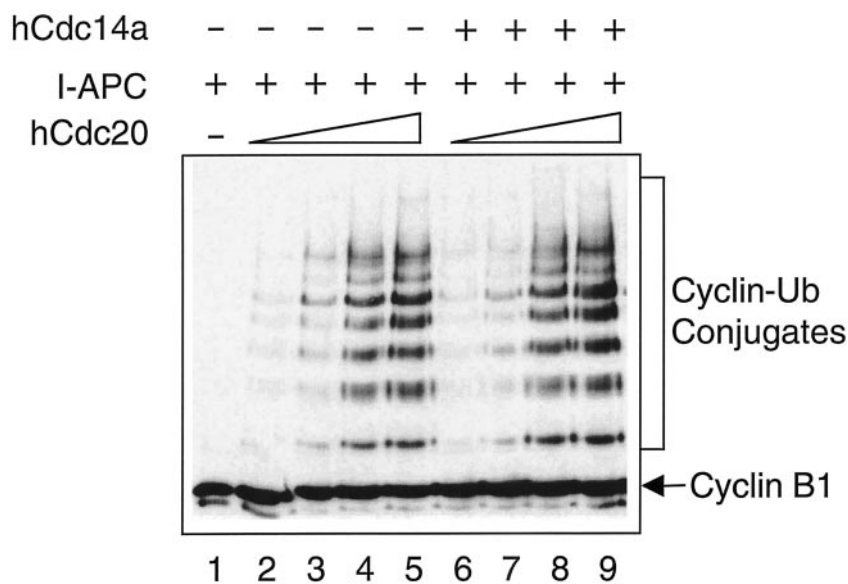
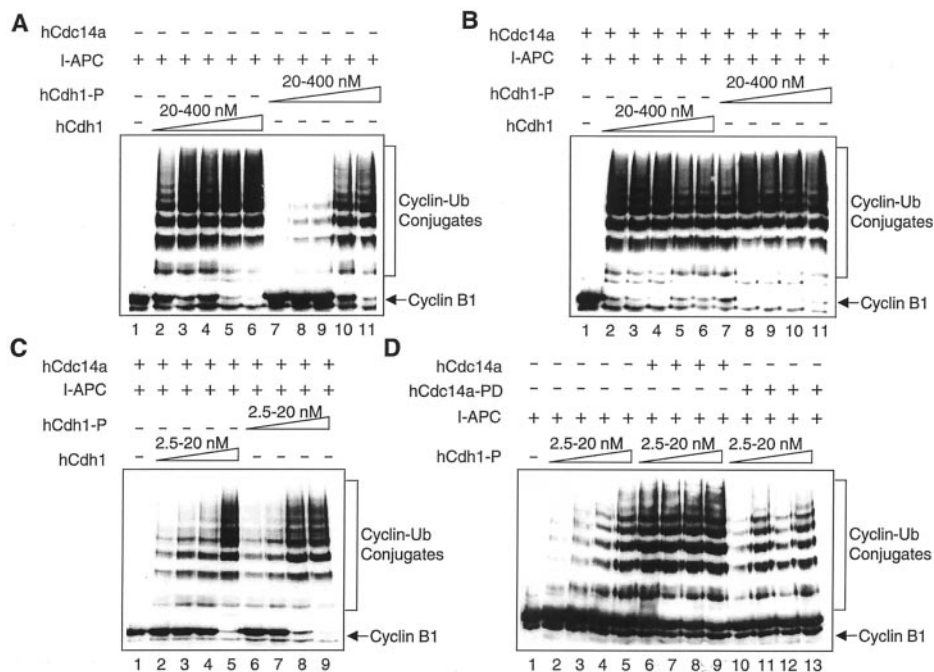


FIG. 4. hCdc14a does not affect the activity of APC^{Cdc20}. Interphase *Xenopus* APC was incubated with hCdc20, in the absence (lanes 2–5) or presence (lanes 6–9) of hCdc14a and assayed for ubiquitination activity.

hCdc14a Is a Major Phosphatase of hCdh1 in HeLa Cells—We next determined the native size of hCdc14 in HeLa cell lysates synchronized at different cell cycle stages. HeLa cells were synchronized at the G₁/S boundary by a double thymidine block and released into fresh medium. Samples were taken at various time points. Based on fluorescence-activated cell sorting analysis and cyclin B1 immunoblotting, cells were effectively synchronized (data not shown). The cell lysates were fractionated on a gel filtration column, and the resulting fractions were then blotted with the anti-hCdc14 antibody (Fig. 5A). In all cell cycle stages, the majority of hCdc14 eluted in fractions 14 and 15, which corresponded to a molecular mass of about 500 kDa. We then concentrated the fractions of the G₁ phase lysate (12 h after the release of the thymidine block) and assayed their ability to dephosphorylate hCdh1N. A final concentration of 2 μ M of microcystin was added to inhibit PP1 and PP2A in these fractions. The phosphatase activity that dephosphorylates hCdh1N co-eluted

with hCdc14a, with the exception of fraction 17 (Fig. 5B). Therefore, hCdc14a co-elutes with a major hCdh1 phosphatase in HeLa cell lysates.

hCdc14a Localizes to the Centrosomes—The yeast Cdc14 protein is sequestered by Net1/Cfi1 in the nucleolus (13, 14, 18). The release of Cdc14 from the nucleolus at late anaphase then activates APC^{Cdh1}, leading to the exit from mitosis (13, 14). To determine whether a similar regulatory mechanism exists in mammals, we examined the localization of hCdc14 in HeLa cells by indirect immunofluorescence. The affinity-purified polyclonal α -hCdc14a antibody recognized a predominant band of 68 kDa in HeLa cell lysate (Fig. 6A). The predicted molecular mass of hCdc14a is 69.6 kDa. Therefore, the α -hCdc14a antibody specifically detects hCdc14a in HeLa cell lysate. HeLa cells were then fixed with paraformaldehyde, permeabilized, and stained with the α -hCdc14a antibody. In addition to the nuclear staining, hCdc14 also localized to the centrosomes in interphase cells (Fig.

FIG. 5. hCdc14a exists as a part of a 500-kDa complex in HeLa cells. *A*, the lysate of HeLa cells synchronized with a double thymidine block was fractionated on a 26-ml Superose 6 column. The upper panel shows the UV traces of all four column runs. All four traces were virtually identical, indicating that there was little variation between the column runs. The lower panel shows the α -hCdc14a immunoblots of column fractions. The estimated molecular weight of the peak fractions is indicated. *B*, the fractions of the lysate made from cells 12 h after thymidine removal were concentrated and assayed for their ability to dephosphorylate hCdh1N in the presence of microcystin.

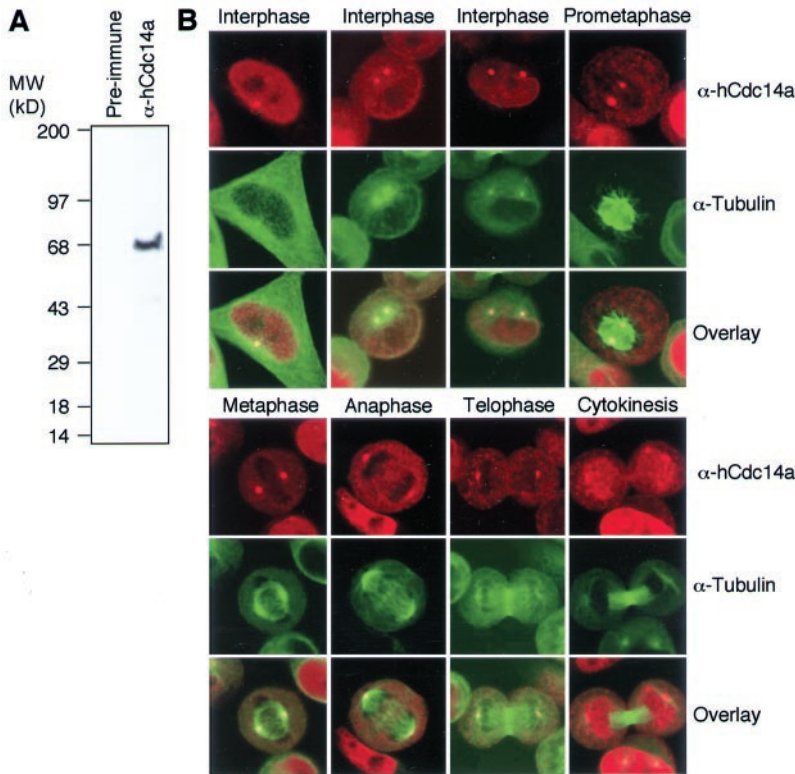
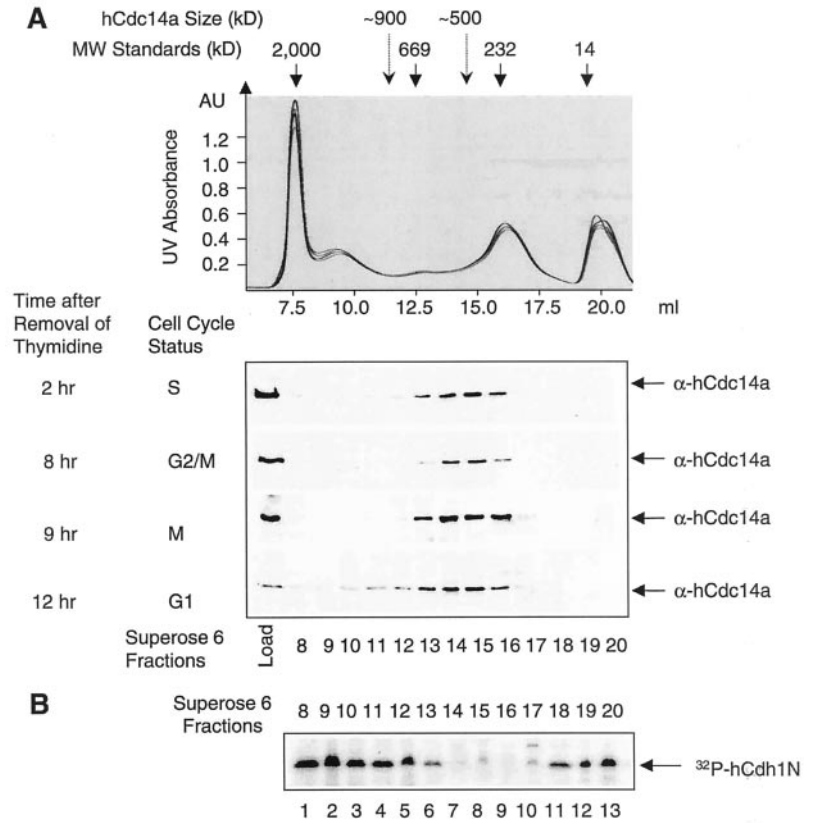


FIG. 6. hCdc14a localizes to centrosomes in HeLa cells. *A*, HeLa cell lysate was separated by SDS-PAGE and blotted with either the preimmune serum or the affinity-purified α -hCdc14a antibody. *B*, HeLa cells at various cell cycle stages were double-stained with α -hCdc14a (in red) and α -tubulin (in green) antibodies.

6B). The centrosomal localization of hCdc14 persisted in all phases of mitosis (Fig. 6B). We did not observe clear nucleolus staining of hCdc14a at any stages of the cell cycle.

DISCUSSION

Conversion from APC^{Cdc20} to APC^{Cdh1} in Late Anaphase—APC controls two critical transitions during the cell cycle: sis-

ter-chromatid separation and the exit from mitosis by mediating the sequential degradation of securins and mitotic cyclins. The degradation of securins should always precede that of cyclin B and other APC substrates, such as Cdc5/Plk (8, 28, 29) and Cdc20 itself (11), that are essential for various late mitotic events. Therefore, the timing of degradation of these APC sub-

strates has to be carefully orchestrated. The timely degradation of the APC substrates depends on the proper regulation of the APC activity, especially the conversion from APC^{Cdc20} to APC^{Cdh1} in late anaphase. Phosphorylation of APC and Cdh1 plays an important role in controlling this conversion. In mitosis, phosphorylation of several APC subunits enhances the activity of APC^{Cdc20} (8, 25). On the other hand, phosphorylation of hCdh1 inhibits its ability to stimulate APC. This is essential for keeping APC^{Cdh1} off until cyclin B-Cdc2 has completed its functions in mitosis. Our results reported herein indicate that, similar to the regulation of APC^{Cdh1} in budding yeast, dephosphorylation of hCdh1 by hCdc14a leads to the activation of APC^{Cdh1} at late anaphase in mammalian cells.

In addition to phosphorylation, both APC^{Cdc20} and APC^{Cdh1} are also regulated by Mad2 and its related protein Mad2b, in response to the mitotic checkpoint signals (30–32). It remains to be determined whether and how phosphorylation of hCdh1 and Mad2b binding cooperate to inhibit APC^{Cdh1} in late anaphase.

Localization and Regulation of hCdc14a—In yeast, a major mechanism for regulating the function of Cdc14 is to control its cellular localization (13, 14). Prior to late anaphase, Cdc14 is anchored in the nucleolus by the RENT protein complex containing Net1/Cfi1. It is the release of Cdc14 from the nucleolus that allows Cdc14 to encounter its substrate Cdh1 and activate APC^{Cdh1}. However, hCdc14a does not appear to localize to the nucleolus in HeLa cells. Instead, aside from the diffusive nuclear distribution, hCdc14a localizes to the centrosomes. Spatial separation between hCdc14a and its substrate might not be the mechanism of regulation for hCdc14a in mammalian cells. In any case, nucleolus sequestration is not expected to be the sole mechanism for the regulation of the vertebrate Cdc14, simply because the nucleoli in vertebrate cells dissolve in prometaphase long before the activation of APC^{Cdh1}. It is also worth noting that no Net1/Cfi1 homologs have been identified in vertebrates so far.

The localization pattern of hCdc14a is consistent with its role in regulating APC^{Cdh1} *in vivo* for several reasons. In *Drosophila*, the degradation of cyclin B is spatially regulated; the spindle-associated cyclin B is degraded first at the spindle poles, and the degradation then spreads toward the equator, whereas the cytosolic cyclin B is degraded at an even later time (33). In mammalian cells, cyclin B1 degradation also occurs first at the spindle poles and chromosomes (34). Several APC subunits are enriched at centrosomes (35). Therefore, the localization of hCdc14a, an activator of APC^{Cdh1}, at the centrosomes provides the potential explanation for the initiation of cyclin B degradation at the spindle poles. In addition, several MEN proteins, such as Dbf2 and Cdc15, and the negative regulator of the MEN pathway, Bub2, localize to spindle poles in budding yeast (36–39). The centrosome localization of hCdc14a suggests that the MEN proteins in mammalian cells might also be enriched at the centrosomes. The lack of nucleolus sequestration of

hCdc14a in HeLa cells suggests that, despite the conservation of some of the molecular players of the mitotic exit network, the MEN proteins might utilize distinct mechanisms to regulate hCdc14a in mammalian cells.

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