Activation of the human anaphase-promoting complex by proteins of the CDC20/Fizzy family

Edgar R. Kramer*, Christian Gieffers*, Gabriele Hölzl†, Markus Hengstschläger† and Jan-Michael Peters*

The initiation of anaphase and exit from mitosis depend on the activation of the cyclosome/anaphase-promoting complex (APC) that ubiquitinates regulatory proteins such as anaphase inhibitors and mitotic cyclins [1–4]. Genetic experiments have demonstrated that two related WD40-repeat proteins – called Cdc20p and Hct1p/Cdh1p in budding yeast and Fizzy and Fizzy-related in *Drosophila* – are essential for APC-dependent proteolysis [5–11]. Human orthologs of these proteins – hCDC20/p55^{CDC} [12] and hCDH1 – have recently been found to associate with APC in a cell-cycle-dependent manner [13,14]. Here, we show that the amount of hCDC20 and hCDH1 bound to APC correlates with a high ubiquitination activity of APC and that binding of recombinant hCDC20 and hCDH1 can activate APC *in vitro*. Our results suggest that the association between hCDH1 and APC is regulated by post-translational mechanisms, whereas the amount of hCDC20 bound to APC may in addition be controlled by hCDC20 synthesis and destruction [15]. The temporally distinct association of hCDC20 and hCDH1 with APC suggests that these proteins are, respectively, mitosis-specific and G1-specific activating subunits of APC.

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Results and discussion

APC regulation during the human somatic cell cycle

To address whether APC activity is regulated by association with hCDC20 or hCDH1, we enriched HeLa cells in different cell-cycle phases by drug treatment and analyzed the steady-state levels of hCDC20, hCDH1 and APC (Figure 1a), the amounts of hCDC20 and hCDH1 bound to APC (Figure 1b), and the specific cyclin B ubiquitination activity associated with APC (Figure 1c). As reported recently [15], hCDC20 levels increased between G1 and mitosis. Similar amounts of hCDH1 and the APC subunits CDC27 and APC2 were detectable in all cell-cycle fractions but the electrophoretic mobility of hCDC27, hCDH1 and hCDC20 was decreased in mitotic cells (Figure 1a). These mobility shifts could not be detected after treatment with λ protein phosphatase, indicating that these proteins are phosphorylated in mitosis.

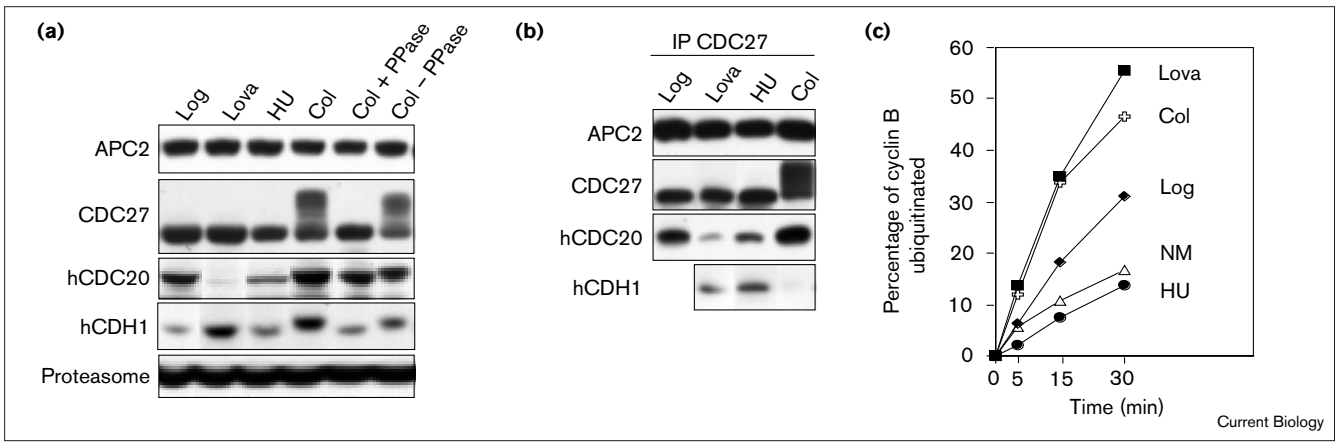
Density gradient centrifugation and co-immunoprecipitation experiments indicated that a fraction of hCDC20 and hCDH1 is bound to APC (see the Supplementary material published with this paper on the internet). In contrast to a report [16] that *Xenopus* CDC20/Fizzy does not associate with APC, our results agree with recent reports ([13,14]; W. Zachariae and K. Nasmyth, personal communication) that human and yeast CDC20 and CDH1 co-immunoprecipitate with APC. The APC isolated from G1 cells contained only small amounts of hCDC20, whereas increasingly larger amounts co-immunoprecipitated from S-phase and mitotic cells, respectively (Figure 1b). In contrast, similar amounts of hCDH1 were bound to APC in G1 and S phases, and hardly any hCDH1 was associated with APC in mitosis. APC's specific cyclin B ubiquitination activity was significantly higher in mitosis and G1 than in S or G2 (Figure 1c), demonstrating that APC activity is regulated in somatic cell cycles, as is the case in embryonic cell cycles [2,3].

Some of the drugs used in the above experiments interfere with APC regulation by activating the spindle checkpoint ([13,14] and references therein). To analyze APC regulation in the absence of cell-cycle inhibitors we therefore fractionated HeLa cells by centrifugal elutriation. As in drug-arrested cells, the amount of hCDC20 associated with APC increased between G1 and G2/M, whereas the binding of hCDH1 to APC decreased between G1 and G2/M, despite constant hCDH1 steady-state levels (Figure 2a,b). In cyclin B ubiquitination assays, APC activity was maximal in G1 (Figure 2c and Supplementary material). In both drug-arrested and elutriated cells the amount of hCDC20 bound to APC therefore correlates with hCDC20 steady-state levels, whereas the binding of hCDH1 to APC is cell-cycle regulated. Importantly, these data show that the activity of APC is high in mitosis and G1 when large amounts of either hCDC20 or hCDH1 are associated with APC.

hCDC20 and hCDH1 activate APC *in vitro*

To test whether the binding of hCDC20 and hCDH1 activates APC, we synthesized radiolabeled hCDC20 and

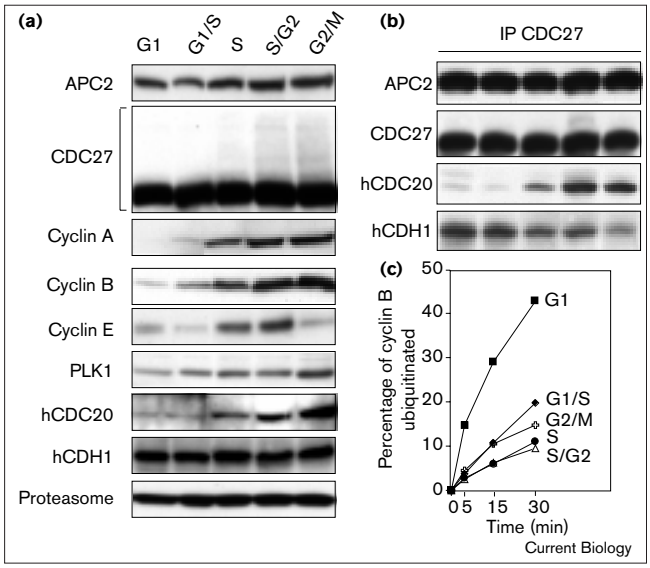
Figure 1



APC regulation in drug-arrested HeLa cells. **(a)** Extracts of logarithmically growing cells (log) and cells enriched either in G1 with lovastatin (lova), in S with hydroxyurea (HU) or in mitosis with colcemid (col) were analyzed by immunoblotting with antibodies specific to the proteins indicated on the left. In addition, lysates from colcemid-arrested cells were either incubated with λ protein phosphatase (col + PPase) or with control buffer (col – PPase) for 30 min at 30°C before immunoblot analysis. Equal amounts of proteins were analyzed in each lane. Anti-proteasome antibodies were used as a loading control. **(b)** APC immunoprecipitates (IPs) were analyzed by immunoblotting as in (a). **(c)** Time course of ubiquitination of ^{125}I-labeled cyclin B 13–110 by APC immunoprecipitates in a reconstituted system. Nitrogen mustard (NM) was used to enrich cells in G2.

hCDH1 by transcription–translation reactions and tested the ability of these proteins to bind to APC and to modulate its activity. As a control, we used the β subunit of protein phosphatase 2A (PP2Aβ). Like hCDC20 and hCDH1, PP2Aβ is a 55 kDa protein that contains seven WD40 repeats (E. Ogris and A. Bauer, personal communication). A significant amount of hCDH1 and hCDC20 bound to immunoprecipitated APC, whereas only a small amount of these proteins bound to antibody beads in the absence of APC (Figure 3) or to pre-immune beads (unpublished observation). PP2Aβ was only slightly enriched on APC beads compared with control beads. Similar amounts of hCDC20 associated with APC isolated from cells in logarithmic, G1 or S phase, but about three times as much hCDC20 bound to mitotic APC (Figure 3). A similar result was obtained for hCDH1, whereas the small amount of PP2Aβ recovered on APC beads did not vary in a cell-cycle-dependent manner. The binding of hCDH1 to APC correlated with a significant increase in APC's cyclin B ubiquitination activity that was not observed when APC was pre-incubated with reticulocyte lysate alone (Figure 4a,b). Also, the binding of hCDC20 to APC correlated with an increase in APC activity, but this effect was significantly smaller. PP2Aβ had no effect on APC activity. hCDH1 could also bind to *Xenopus* APC, the activity of which was thereby dramatically increased (Figure 4c,d). In dose–response experiments, APC activity correlated with the amount of associated hCDH1 (Supplementary material), implying that the binding of hCDH1 and the stimulation of APC activity are causally related.

Figure 2

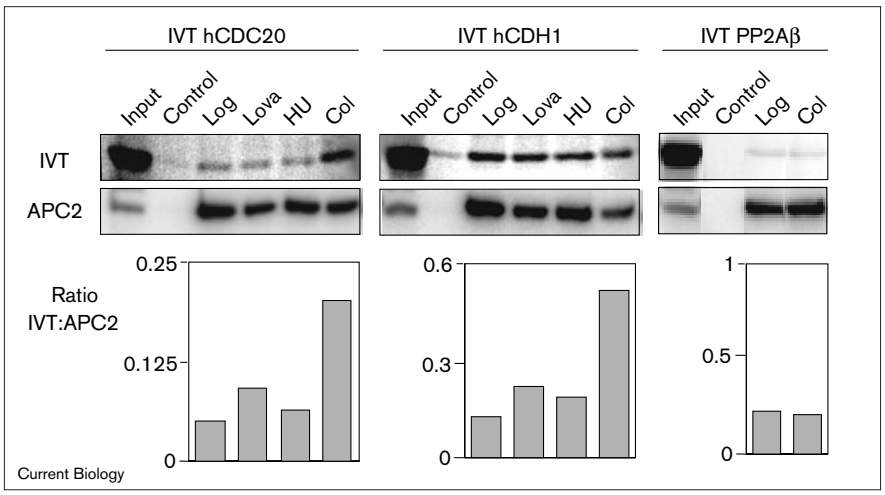


APC regulation in elutriated HeLa cells. **(a)** Immunoblot analysis of lysates from elutriated cells. **(b)** APC immunoprecipitates obtained were analyzed by immunoblotting as in (a). **(c)** Time course of ubiquitination of ^{125}I-labeled cyclin B 13–110 by APC immunoprecipitates in a reconstituted system.

How do hCDC20 and hCDH1 activate APC-dependent proteolysis?

Our results, and similar experiments that were published by Fang *et al.* [17] after submission of this manuscript, suggest that the amounts of hCDC20 and hCDH1 bound to APC

Figure 3



In vitro translated hCDC20 and hCDH1 bind to APC from HeLa cells. APC immunoprecipitates were incubated with reticulocyte lysates containing *in vitro* translated (IVT) hCDC20, hCDH1 or PP2Aβ, which were labeled with [^{35}S]methionine and [^{35}S]cysteine. As a specificity control, CDC27 antibody beads were used without bound APC. Subsequently, the immunoprecipitates were washed in buffers containing 0.5 M KCl and 5% NP40 and analyzed by SDS–PAGE and phosphorimaging. The amount of APC in each immunoprecipitate was determined by quantitative immunoblotting with APC2 antibodies and ^{125}I-labeled secondary antibodies. The intensities of the *in vitro* translated proteins are not directly comparable because hCDC20, hCDH1 and PP2Aβ contain 14, 13 and 19 ^{35}S-labeled residues, respectively.

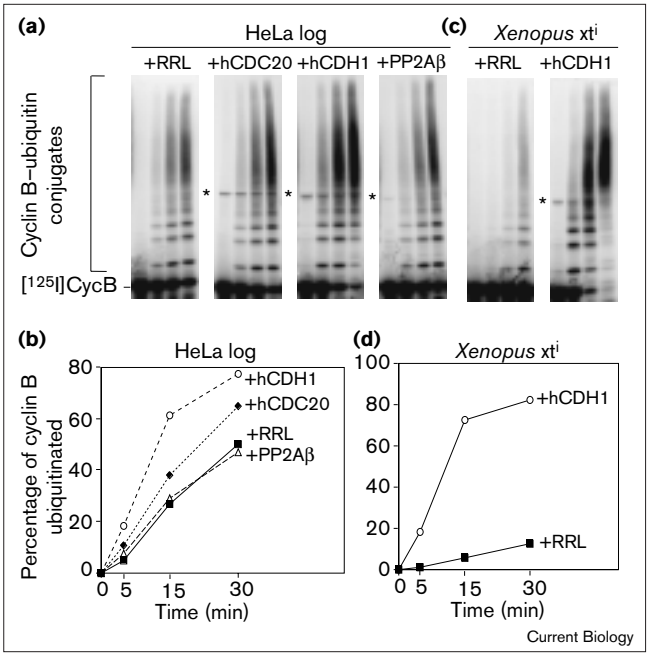
are rate-limiting for ubiquitination reactions. We do not know, though, whether hCDC20 and hCDH1 participate directly in substrate ubiquitination or allosterically activate other APC components. In this respect it is worth noting that hCDC20 and hCDH1 contain WD40 repeats, as do several substrate-recognition factors of the SCF ubiquitination complex [4]. It is therefore conceivable that hCDC20 and hCDH1 function by recruiting specific substrates to APC, a hypothesis that could explain why Cdc20p and Hct1p/Cdh1p confer substrate specificity to APC-dependent proteolysis in yeast [7,9,11]. It will be interesting to test whether hCDC20 and hCDH1 bind APC substrates in a specific manner. Our observation that both hCDC20 and hCDH1 could stimulate cyclin B ubiquitination *in vitro* does not rule out the possibility that hCDC20 and hCDH1 display specificity towards other APC substrates.

How are hCDC20 and hCDH1 regulated?

Unlike previously identified APC subunits, hCDC20 and hCDH1 bind to APC preferentially during mitosis and G1, respectively, suggesting that hCDC20 is required for mitotic APC activity and hCDH1 may maintain APC activity in G1. This interpretation agrees well with the observation that the expression of *Drosophila* and *Xenopus* CDH1/Fizzy-related is restricted to cell cycles that include a G1 phase, in contrast to the expression of CDC20/Fizzy, which is essential for mitosis in embryonic cell cycles [8,16].

Although our results are consistent with the view that binding of hCDC20 to APC is limited by the cellular levels of hCDC20, additional mechanisms may regulate the association between hCDC20 and APC. The latter possibility is suggested by our observation that only a fraction of hCDC20 is associated with APC (Supplementary material) and by the finding that mitotic APC binds more recombinant hCDC20 than APC from other cell-cycle phases (Figure 3). Mitotic phosphorylation of APC or

Figure 4



hCDC20 and hCDH1 activate APC *in vitro*. APC immunoprecipitates from logarithmic HeLa cells (log) or from *Xenopus* interphase extracts (xt^{i}) were incubated with reticulocyte lysates containing either hCDC20, hCDH1, PP2Aβ, or no translation product (+RRL). Subsequently, the immunoprecipitates were washed and used in ubiquitination assays. Samples were taken at 0, 5, 15 and 30 min and analyzed by SDS–PAGE and phosphorimaging **(a,c)**, and activities were quantitated **(b,d)**. The *in vitro* translated proteins associated with APC are marked by asterisks.

hCDC20 may therefore increase their association, a possibility that could explain the previously observed requirement for phosphorylation in mitotic APC activation [3,18–20].

Our results indicate that the binding of hCDH1 to APC is inhibited in mitosis. It is unlikely that mitotic APC phosphorylation prevents hCDH1 binding because recombinant hCDH1 associated well with mitotic APC (Figure 3); however, hCDH1 is also subject to phosphorylation *in vivo* (Figure 1a), and it will therefore be interesting to test whether this modification results in dissociation of hCDH1 from APC. Phosphorylation of hCDH1 may also prevent hCDH1 binding in S and G2, where less hCDH1 is associated with APC. This hypothesis could explain why mitotic as well as G1-specific kinases inhibit Hct1p/Cdh1p-dependent proteolysis in yeast [21] and why cyclin E/CDK2 negatively regulates degradation of mitotic cyclins in *Drosophila* [8].

In summary, our results suggest that hCDC20 and hCDH1 are rate-limiting activating subunits of APC in mitosis and G1, respectively. The amount of hCDC20 bound to APC is limited by the cellular steady-state levels of hCDC20, but efficient binding in mitosis may further require mitotic phosphorylation of APC or hCDC20. hCDH1 is present at constant levels throughout the cell cycle but appears to associate with APC only from the end of mitosis or the beginning of G1. The binding of hCDH1 to APC is decreased in S and G2 phase and completely inhibited in early mitosis, perhaps by phosphorylation of hCDH1. These results indicate that the binding of hCDC20 and hCDH1 to APC is a regulated process that controls APC activity during the cell cycle.

Supplementary material

Additional methodological detail and three additional figures are published with this article on the internet.

Acknowledgements

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Supplementary material

Activation of the human anaphase promoting complex by proteins of the CDC20/Fizzy family

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Materials and methods

cDNAs and recombinant protein expression

Sequence analysis revealed that the human expressed sequence tag clones 577261 and 124782 (nomenclature of the American Type Culture Collection (ATCC)) represent overlapping 5′ and 3′ fragments of a human cDNA highly related to *Xenopus fizzy-related* and budding yeast *HCT1/CDH1* [S1–S3]. A complete hCDH1 cDNA (pHCDH1-BS) was constructed by cloning the non-overlapping parts of both fragments into pBluescript (Stratagene). This cDNA encodes a protein of 493 amino acid residues with a predicted molecular mass of 55 kDa. Sequence analysis showed that the ATCC clone number 551326 contains a human cDNA encoding a protein identical to $p55^{CDC}$ [S4] except for amino acid residue 101 which is a serine in $p55^{CDC}$ but a proline in our sequence. We refer to this cDNA as hCDC20. The National Center for Biotechnology Information (NCBI) accession number for hCDC20 is AF099644. *Xenopus fizzy-related* [S1] and rat *PP2Aβ* cDNAs [S5] were kindly provided by C. Lehner and E. Ogris, respectively.

[^{35}S]methionine-labeled and [^{35}S]cysteine-labeled proteins were prepared by coupled transcription–translation reactions in rabbit reticulocyte lysate (Promega). Bacterially synthesized hCDH1 and hCDC20 were generated by cloning the respective cDNAs into pET-28 vectors (Novagene). Proteins containing an amino-terminal hexahistidine tag were expressed in *Escherichia coli* BL21(DE)pLysS cells (Novagene) and purified on Ni–NTA agarose (Qiagen).

Antibodies

Antibodies to human cyclins A, B and E and hCDC20 (carboxy-terminal peptide) were from Santa Cruz. Additional hCDC20 antibodies were raised against bacterially synthesized protein in rabbits. Antibodies against hCDH1 were generated by immunizing rabbits with the synthetic peptide MDQDYERRLLRQINLQNEC (amino terminus of *Xenopus* Fizzy-related) coupled to keyhole limpet hemocyanin. In immunoblotting experiments, the resulting hCDH1 antisera recognized bacterially expressed hCDH1, *in vitro* translated hCDH1 and *Xenopus* Fizzy-related and a 55 kDa band in HeLa extracts. No crossreaction was observed in immunoblotting experiments between hCDH1 antibodies and hCDC20 protein, or between hCDC20 antibodies and hCDH1 protein. APC and PLK1 antibodies were generated by immunizing rabbits with recombinant proteins. Monoclonal anti-proteasome antibodies have been described [S6].

Cell culture and cell synchronization

Adherent HeLa cells were grown at 37°C and in the presence of 5% CO_2 in DMEM supplemented with 10% fetal bovine serum, 0.3 μg/ml L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. To induce cell-cycle arrests, cells were grown to 50% confluency and subsequently cultured for 17–18 h in the presence of 330 nM nocodazole (Sigma), 190 nM colcemid (Sigma), 50 μM activated lovastatin (Merck Sharp and Dohme), 2 mM hydroxyurea (Sigma), 400 μM mimosine (Aldrich) or 10 μM nitrogen mustard (Sigma). Lovastatin was activated as described [S7].

Separation of logarithmically growing cells into distinct cell-cycle phases was accomplished by centrifugal elutriation which was performed as described [S8], using a Beckman J2–21 M centrifuge equipped with a JE–6B rotor and a standard separation chamber. The rotor was kept at a speed of 2000 rpm and a temperature of 20°C, and medium flow was controlled with a Cole-Parmer Masterflex pump. Typically, 8×10^8 cells were fractionated, and consecutive fractions of 150 ml were collected at increasing flow rates. Cytofluorometric analyses were performed using a Becton–Dickinson FACScan. DNA was stained with propidium iodide (Sigma).

Preparation of cell extracts and protein fractionation

HeLa cells arrested with drugs were scraped from the plates and washed with ice-cold PBS (140 mM NaCl, 6.4 mM Na_2HPO_4, 2.6 mM KCl, 1.4 mM KH_2PO_4, pH 7.4). Elutriated cell fractions were also washed with PBS before lysis. Protein extracts were prepared in buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 10% glycerol, 2 mM EDTA, pH 8.0, 50 mM NaF, 0.25 mM Na_3VO_4, 1 mM PMSF, 1 mM DTT, 1 μM okadaic acid (Calbiochem), and 10 μg/ml each of chymostatin, leupeptin, and pepstatin (Sigma). After 20 min on ice with occasional douncing or pipetting, the lysates were centrifuged at 14,000 rpm in an Eppendorf centrifuge, shock frozen in liquid nitrogen and stored at −70°C. *Xenopus* interphase egg extracts were prepared as described [S9].

For sucrose density gradient centrifugation, 10–40% sucrose gradients loaded with cell extracts containing 2–3 mg protein were centrifuged for 18 h at 30,000 rpm in an SW40 rotor (Beckman) at 4°C. Gradients were prepared with a Biocom gradient master and fractions were collected with a density gradient fractionator (ISCO).

Immunoprecipitation and immunoblotting experiments

For the immunoblot analysis of immunoprecipitates, CDC27 antibodies were coupled to protein A-Affiprep beads (Bio-Rad) for 2 h at 4°C and crosslinked with dimethylpimilimidate as described [S10]. CDC27 antibody beads (20 μl) were incubated with 4–5 mg protein diluted in IP buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 mM β-glycerophosphate, 5% glycerol, 0.01% NP-40, 0.5 mM DDT, proteinase inhibitors ['complete mixture', Boehringer]) for 2 h at 4°C. Subsequently, the beads were washed four times with immunoprecipitation buffer and bound proteins were eluted with 100 mM glycine-HCl pH 2.0 and analyzed by SDS–PAGE and immunoblotting using PVDF membrane. Immunoreactions were detected using the enhanced chemiluminescence system (Amersham).

In vitro *binding assays*

To analyze the binding of *in vitro* translated proteins to the APC, 6 μl CDC27 antibody beads were washed with QA buffer (10 mM Tris-HCl pH 7.5, 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM DTT) and incubated either with HeLa cell extract containing 3 mg protein or with QA buffer for 2 h at 4°C. Beads were washed once with 200 volumes QA, once with QA containing additional 400 mM KCl and 5% NP-40, and three times with QA. *In vitro* translation mixtures were added to the APC beads and incubated for 30 min at 37°C. The APC beads were then washed five times as above and bound proteins were eluted with SDS sample buffer, separated by SDS–PAGE and analyzed by phosphorimaging or immunoblotting using ^{125}I-labeled secondary antibodies. Radiolabeled gels were analyzed on a phosphorimager and quantitated using the ImageQuant program (Molecular Dynamics).

In vitro *ubiquitination assays*

To measure APC activity, 6 μl CDC27 antibody beads were washed with QA buffer and incubated either with HeLa cell extract containing 3 mg protein or with *Xenopus* egg extract containing 1 mg protein for 2 h at 4°C. Subsequently, the beads were washed as described for the

in vitro binding assays. Where indicated, APC beads were pre-incubated with *in vitro* translation mixtures for 30 min, either at 37°C in the case of HeLa APC or at room temperature in the case of *Xenopus* APC. The APC beads were then washed again five times as above. Ubiquitination assays were performed in a volume of 10 µl, containing 6 µl beads and 4 µl reaction mix (80 µg/ml bacterially expressed and purified wheat E1, 50 µg/ml each of *Xenopus* UBC4 and UBCx, 1.25 mg/ml ubiquitin, 30 units/ml rabbit creatine phosphokinase type I (Sigma), 7.5 mM creatine phosphate, 1 mM ATP, 1 mM $MgCl_2$, 0.1 mM EGTA, and 3 µg/ml iodinated sea-urchin cyclin B fragment (amino acids 13–110) as a substrate). Reaction mixtures containing HeLa cell APC were incubated in an Eppendorf thermomixer at 37°C; mixtures containing *Xenopus* APC were incubated at room temperature. Samples were analyzed by 5–15% SDS–PAGE, phosphorimaging and quantitation as described above.

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S3. Visintin R, Prinz S, Amon A: **CDC20 and CDH1: a family of substrate-specific activators of APC-dependent proteolysis.** *Science* 1997, **278:**460-463.

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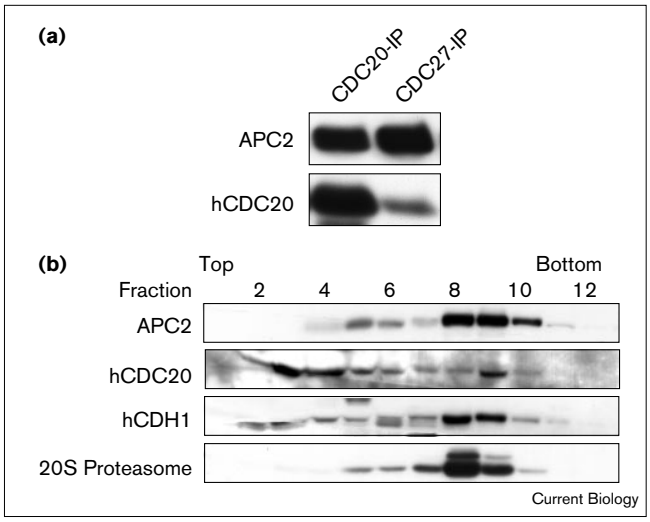
Figure S1



Association of hCDC20 and hCDH1 with APC.
(a) Co-immunoprecipitation of APC subunits and hCDC20. Extracts from logarithmically growing cells were precipitated with antibodies either against hCDC20 (CDC20-IP) or against CDC27 (CDC27-IP) and the immunoprecipitates were analyzed by immunoblotting against APC2 and hCDC20. Note that much less hCDC20 coprecipitates with CDC27 than with hCDC20 antibodies, suggesting that only a small fraction of APC complexes are associated with hCDC20.
(b) Cosedimentation of hCDH1 and hCDC20 with APC during sucrose density gradient centrifugation. Extracts from logarithmically growing cells were fractionated on a 10–40% sucrose gradient. Subsequently, each fraction was analyzed by immunoblotting with antibodies to the indicated proteins. The centrifugation was performed under conditions that destabilize the 26S proteasome holo-complex (see [S6]). Therefore, the proteasome antibodies detect only the 20S core complex of the 26S proteasome.

Figure S2

Regulation of APC's cyclin B ubiquitination activity in elutriated HeLa cells. **(a)** Analysis of the cellular DNA content of elutriated cells by flow cytometry. **(b,c)** Time courses showing the ability of APC immunoprecipitates from elutriated cells to ubiquitinate ^{125}I-labeled cyclin B (CycB) in a reconstituted system. Samples were analyzed by SDS–PAGE and phosphorimaging (c), and the ubiquitination activities were expressed as the percentage of cyclin B converted into conjugates (b). **(d)** Quantitative immunoblot analysis of the immunoprecipitates used in (b,c) with APC2 antibodies and ^{125}I-labeled secondary antibodies. Immunoreactions were detected by phosphorimaging. Quantitation of the immunoreactions showed that the amounts of APC detected in each immunoprecipitate varied by less than 10% (data not shown).

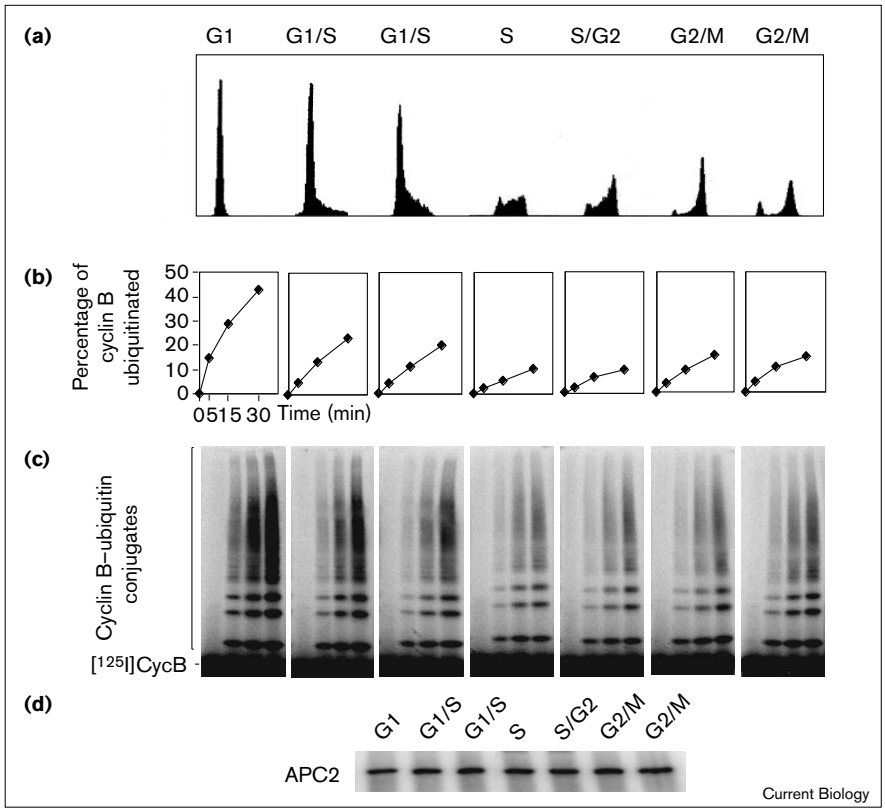


Figure S3

Dose-dependent activation of APC's cyclin B ubiquitination activity by *in vitro* translated hCDH1. **(a,b)** Time courses showing the ability of APC immunoprecipitates associated with different amounts of hCDH1 to ubiquitinate ^{125}I-labeled cyclin B (CycB) in a reconstituted system. APC was isolated from logarithmically growing HeLa cells (log) and incubated with different volumes of reticulocyte lysates containing either *in vitro* translated hCDH1 (+hCDH1), or no translation product (+RRL). Subsequently, the immunoprecipitates were re-isolated, washed and used in ubiquitination assays. Samples were analyzed by SDS–PAGE and phosphorimaging (b), and the ubiquitination activities were expressed as the percentage of cyclin B converted into conjugates (a). *In vitro* translated hCDH1 associated with the APC is marked by asterisks. **(c)** Quantitation of the different amounts of *in vitro* translated hCDH1 associated with APC.