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TPR Subunits of the Anaphase-Promoting Complex Mediate Binding to the Activator Protein CDH1

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Summary

Background: Chromosome segregation and mitotic exit depend on activation of the anaphase-promoting complex (APC) by the substrate adaptor proteins CDC20 and CDH1. The APC is a ubiquitin ligase composed of at least 11 subunits. The interaction of APC2 and APC11 with E2 enzymes is sufficient for ubiquitination reactions, but the functions of most other subunits are unknown.
Results: We have biochemically characterized subcomplexes of the human APC. One subcomplex, containing APC2/11, APC1, APC4, and APC5, can assemble multiubiquitin chains but is unable to bind CDH1 and to ubiquitinate substrates. The other subcomplex contains all known APC subunits except APC2/11. This subcomplex can recruit CDH1 but fails to support any ubiquitination reaction. In vitro, the C termini of CDC20 and CDH1 bind to the closely related TPR subunits APC3 and APC7. Homology modeling predicts that these proteins are similar in structure to the peroxisomal import receptor PEX5, which binds cargo proteins via their C termini. APC activation by CDH1 depends on a conserved C-terminal motif that is also found in CDC20 and APC10.
Conclusions: APC1, APC4, and APC5 may connect APC2/11 with TPR subunits. TPR domains in APC3 and APC7 recruit CDH1 to the APC and may thereby bring substrates into close proximity of APC2/11 and E2 enzymes. In analogy to PEX5, the different TPR subunits of the APC might function as receptors that interact with the C termini of regulatory proteins such as CDH1, CDC20, and APC10.

Introduction

The anaphase-promoting complex or cyclosome (APC) is a ubiquitin ligase (E3) that controls important transitions in mitosis and helps to establish and maintain the G1 phase of the eukaryotic cell cycle. The main APC targets in mitosis are the anaphase inhibitor securin, whose destruction promotes sister chromatid separation, and B-type cyclins, which have to be degraded to allow exit from mitosis (reviewed in [1, 2]). The APC initiates these proteolytic reactions by mediating the transfer of activated ubiquitin residues from ubiquitin-conjugating (E2) enzymes to substrates, and this transfer results in the formation of multiubiquitin chains. These chains act as recognition signals that target the substrate proteins for proteolysis by the 26S proteasome (reviewed in [3]).

The APC is an unusually complex E3 composed of at least 11 subunits in vertebrates and 13 subunits in yeast [4, 5]. Like many other ubiquitin ligases, the APC contains a small RING finger subunit [4, 6]. This protein, called APC11, is sufficient to support the assembly of multiubiquitin chains in the presence of the E2 enzyme UBC4, although these reactions show reduced substrate specificity [4, 7]. APC11 interacts with a cullin domain in APC2 [8], and recombinant APC11 monomers and APC11/APC2 heterodimers can bind to E2 enzymes [7, 8]. Similar pairs of cullin and RING finger proteins also form the core of the ubiquitin ligase complexes SCF and CBC [2]. Despite this central role in ubiquitination reactions, it remains unclear how these proteins promote ubiquitin transfer.

The function of other APC subunits is less well understood. The crystal structure of the small subunit APC10/Doc1 implies that this protein binds a yet unidentified ligand [9, 10]. Budding yeast Doc1 has furthermore been reported to be required for the processivity of APC-mediated ubiquitination reactions [11] and for the binding of substrates [12]. Human APC10 can bind to APC3/Cdc27 [9], one of four tetratrico peptide repeat (TPR) proteins found in vertebrate APC. TPR domains are thought to mediate protein-protein interactions [13–15] and were discovered in the first identified APC subunits [13, 16, 17]. Although TPR subunits of the APC are essential for viability in yeast [13, 18] and represent the largest group of structurally related proteins within the APC, their precise role has remained unknown.

Despite this already complex architecture, the APC still requires additional activator proteins for substrate ubiquitination (reviewed by [1, 2]). Mitotic phosphorylation of multiple APC subunits allows association with the activator CDC20 (also known as $p55^{CDC}$, Fizzy, or Slp1). In metaphase and anaphase, APC^{CDC20} ubiquitinates B-type cyclins and thereby inactivates cyclin-dependent kinase 1 (CDK1). This CDK1 inactivation in turn leads to activation of a second, CDC20-related APC activator called CDH1 (a.k.a. Fizzy-related, Hct1, Ste9, or Srw1). CDH1 interaction with the APC is restricted to late mitosis and G1/G0, since phosphorylation of CDH1 prevents the assembly of APC^{CDH1} during S, G2, and early mitosis.

CDC20 and CDH1 activate the APC in a substrate-specific manner [19] that depends on the presence of sequence elements called destruction and KEN boxes in APC^{CDC20} and APC^{CDH1} substrates, respectively [20, 21]. CDC20 and CDH1 can furthermore bind to APC substrates (reviewed in [22]) and are required for the association of substrates with the APC [12]. These observations imply that CDC20 and CDH1 are adaptor proteins that recruit substrates to the APC. In budding yeast Cdh1, a conserved sequence element called the C box is required for Cdh1 binding to the APC [23], but it is

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unknown to which APC subunits the activator proteins bind.

To understand how the APC mediates ubiquitination reactions, what the function of its individual subunits is, and how activator proteins interact with the APC, it would be desirable to reconstitute the complex either partly or completely from recombinant subunits. However, with the exception of APC2–APC11 heterodimers [8], it has been impossible to assemble APC subcomplexes to date. We have therefore pursued a reverse strategy and have isolated subcomplexes derived from native human APC and characterized their properties. Combined with structural predictions, our data suggest roles for TPR subunits as receptors for the activators CDH1/CDC20.

Results

The Catalytic Subunits APC2 and APC11 Can Be Reversibly Dissociated from Holo-APC

To identify APC subcomplexes, we searched for conditions that dissociate individual subunits from purified native human APC. Based on previously developed immunopurification methods [24], we treated human APC bound to APC3 antibody beads with various buffer conditions and found that extensive washing with a high-salt sodium phosphate buffer of pH 6.5 greatly reduced the amounts of APC2 and APC11 in the immunoprecipitates. All other APC subunits remained bound to the antibody beads, only the levels of APC10 were slightly reduced. Lowering the pH below 6.3 caused dissociation of all subunits from the antibody beads, presumably because the antibody-antigen interaction was weakened (data not shown). To purify APC2/APC11-depleted complexes, we eluted them with the antigenic APC3 peptide, depleted residual complexes still containing APC2 with APC2 antibodies, and finally reimmunoprecipitated with APC4 antibodies. Silver staining and immunoblotting showed that the remaining APC subunits were still part of an intact complex in which APC2 and APC11 could not be detected (Figure 1A). We refer to this subcomplex as Δ2/11-APC. To confirm that Δ2/11-APC represents an intact macromolecular assembly and not merely a protein aggregate, we analyzed the APC2-depleted eluate by sucrose density gradient centrifugation. The remaining subunits of Δ2/11-APC cosedimented as a peak of approximately 22S, verifying that they were present in a discrete APC subcomplex (see Figure S1 in the Supplemental Data available with this article online). Holo-APC also sediments with an S value of 22 ([24], Figure S1), indicating that the loss of APC2 and APC11 does not grossly change the mass and shape of the remaining complex.

To analyze which functional properties Δ2/11-APC retains, we first tested binding to CDH1. Both holo-APC and Δ2/11-APC could be loaded with comparable amounts of purified recombinant CDH1 (Figure 1B); this finding implies that APC2 and APC11 are dispensable for CDH1 binding. This notion was further supported by the observations that Δ2/11-APC also contained some endogenous CDH1 (Figure 1B), and that CDH1 could neither bind to nor activate recombinant APC2/11 complexes (data not shown). We subsequently analyzed holo-APC and Δ2/11-APC for ubiquitination activity toward the substrate securin in the presence of ATP, CDH1, E1, and the E2 enzymes UBC4 and UBCx. Whereas holo-APC efficiently ubiquitinated securin under these conditions, Δ2/11-APC was completely inactive (Figure 1C).

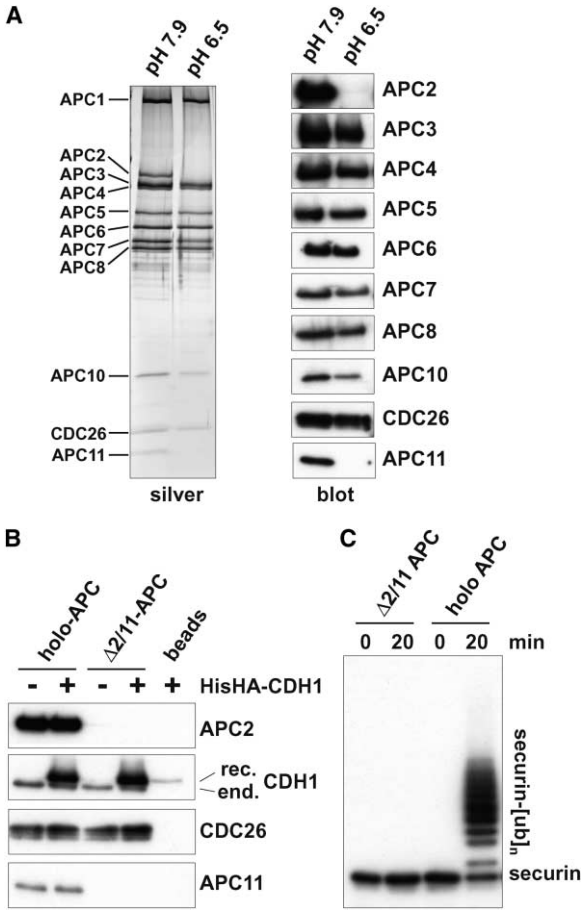

Figure 1. APC2 and APC11 Can Be Dissociated from the APC

(A) A silver-stained gel (left) and Western blot (right) of APC immunoprecipitates after high-salt washes under different pH conditions.

(B) CDH1 binding assay. Holo-APC and Δ2/11-APC were incubated with buffer or recombinant HisHA-CDH1 and were probed with CDH1 antibodies for the presence of endogenous (lower band) and recombinant (upper band) CDH1.

(C) An in vitro ubiquitination assay with holo-APC and Δ2/11-APC. Recombinant securin used as substrate was detected by Western blotting.

The lack of activity associated with Δ2/11-APC could have been caused by the loss of APC2 and APC11 or by damage to other subunits during the mildly acidic pH treatment. To distinguish between these possibilities, we tested if active holo-APC could be reconstituted from purified Δ2/11-APC and APC2/APC11 complexes generated in baculovirus-infected insect cells. Figure 2A shows that coexpressed APC2 and APC11 bound to the depleted complex, whereas little or no association was seen with singly expressed APC2 and APC11, even when a mixture of both proteins was incubated with Δ2/11-APC. This finding is consistent with the observation

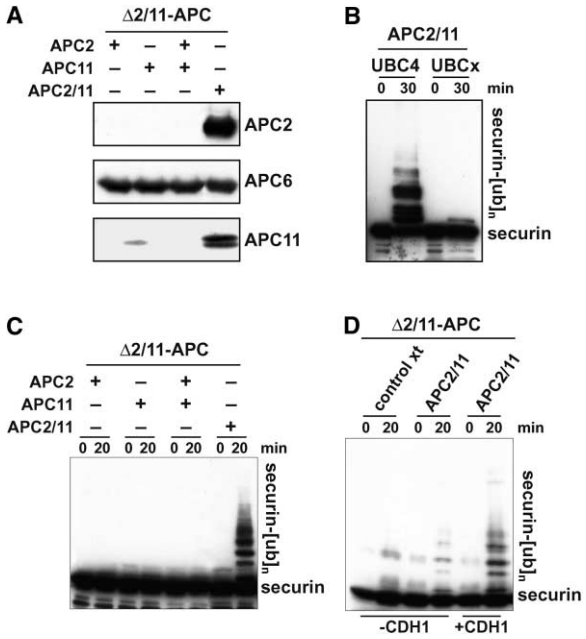

Figure 2. Reconstitution of Active, CDH1-Responsive Holo-APC from Δ2/11-APC and Recombinant APC2 and APC11

(A) A Western blot of Δ2/11-APC bound to αAPC4 beads after incubation with insect cell lysates containing the indicated recombinant proteins.

(B) An in vitro ubiquitination assay with recombinant GST-APC11/APC2 subcomplexes bound to glutathione sepharose and securin in the presence of either UBC4 or UBCx.

(C) An in vitro securin ubiquitination assay with Δ2/11-APC reconstituted with the indicated insect cell lysates in the presence of UBCx and recombinant CDH1.

(D) An in vitro ubiquitination assay with Δ2/11-APC reconstituted with the indicated insect cell lysates in the absence and presence of CDH1.

of Tang et al. [8] that APC2 and APC11 only interact upon coexpression, and further it implies that this interaction either creates a common interface for APC association or is essential for proper folding and structural integrity of these two subunits.

To discriminate a functional interaction from unspecific binding, we examined the ubiquitination activity of the reconstituted complex. Ubiquitination reactions mediated by holo-APC can be supported by either one of the E2 enzymes UBC4 and UBCx in vitro [17, 25], but in our hands, reactions mediated by recombinant APC11 or APC2/11 are only supported efficiently by UBC4 and not by UBCx ([4]; Figure 2B). We therefore used the ability of UBCx to support ubiquitination reactions to discriminate between activity mediated by APC2/11 or by holo-APC. As shown in Figure 2C, Δ2/11-APC that had bound to coexpressed APC2 and APC11 indeed regained the ability to ubiquitinate securin in the presence of UBCx, whereas singly expressed APC2 and/or APC11 could not reactivate Δ2/11-APC. Furthermore, the observed activity was dependent on the activator CDH1 (Figure 2D). Since ubiquitination reactions mediated by APC11 or APC2/11 cannot be stimulated by recombinant CDH1 ([4, 8]; data not shown), this confirmed that active holo-APC had been reconstituted.

This reconstitution of functional holo-APC from Δ2/11-APC and recombinant APC2/11 implies that Δ2/11-APC is not irreversibly damaged by the low-pH treatment, and the lack of ubiquitination activity thus appears to be a direct consequence of the loss of APC2 and APC11. APC2 and APC11 are therefore strictly required for the ubiquitination activity of the APC but are dispensable for CDH1 binding.

APC1, 2, 4, 5, and 11 Form a Stable Subcomplex

We discovered a second APC subcomplex through fractionation of extracts from logarithmically grown HeLa cells on a Source Q15 anion exchange column. Immunoblot analysis of individual fractions showed that holo-APC containing all 11 subunits eluted in a sharp peak at around 450 mM KCl, whereas the TPR proteins APC3, APC6, and APC7 were also found in other fractions, especially in late fractions eluted with higher ionic strength (see Figure S2 in the Supplemental Data). Whereas several APC subunits (e.g., APC10) were only detected in the holo-APC fraction, APC2 and APC11 appeared in a distinct second peak already eluting at 350 mM KCl (Figure S2 and data not shown). To analyze if these proteins were part of a distinct subcomplex or had simply dissociated from holo-APC as individual proteins, we immunoprecipitated APC2 from the 350 mM KCl fraction. Subsequent immunoblotting detected APC1, APC2, APC4, APC5, and APC11 without significant contamination by any other subunit (Figure 3A). To confirm that they form an independent subcomplex, we depleted residual holo-APC from the 350 mM fractions by using APC3 antibodies and subsequently purified the subcomplex with antibodies against APC2, APC4, or APC5. All three antibodies immunoprecipitated the same five subunits in roughly stoichiometric amounts (Figure 3B and data not shown). In an additional purification step, these subunits could also be eluted from APC4 antibody beads by the antigenic peptide and could subsequently be reimmunoprecipitated with APC5 antibodies (Figure 3C). These data indicate that APC1, APC2, APC4, APC5, and APC11 form a stable subcomplex that, based on its elution properties from the Q column, we refer to as Q_{350}-APC.

We presently do not know if Q_{350}-APC was generated during the fractionation, or if it represents a physiological subcomplex, for example, an assembly intermediate. Nevertheless, its apparent stability gave us the opportunity to investigate its properties further. In vitro ubiquitination activity of Q_{350}-APC toward securin was close to that of control reactions containing no E3, despite the presence of APC2/11 (Figure 3D). This was, however, not due to loss of APC2/11 activity, since Q_{350}-APC was capable of multiubiquitin chain assembly (Figure 3E). We also compared the ability of holo-APC and Q_{350}-APC to recruit the E2 enzyme UBCx. After incubation of immunoprecipitated complexes with crosslinker-modified UBCx and subsequent immunoblotting with anti-UBCx antibodies, only a few major bands, whose sizes were consistent with modification of APC2, were observed (Figure 3F). Reprobing with antibodies against APC2 (Figure 3F) and other subunits (data not shown) confirmed that UBCx was specifically crosslinked to

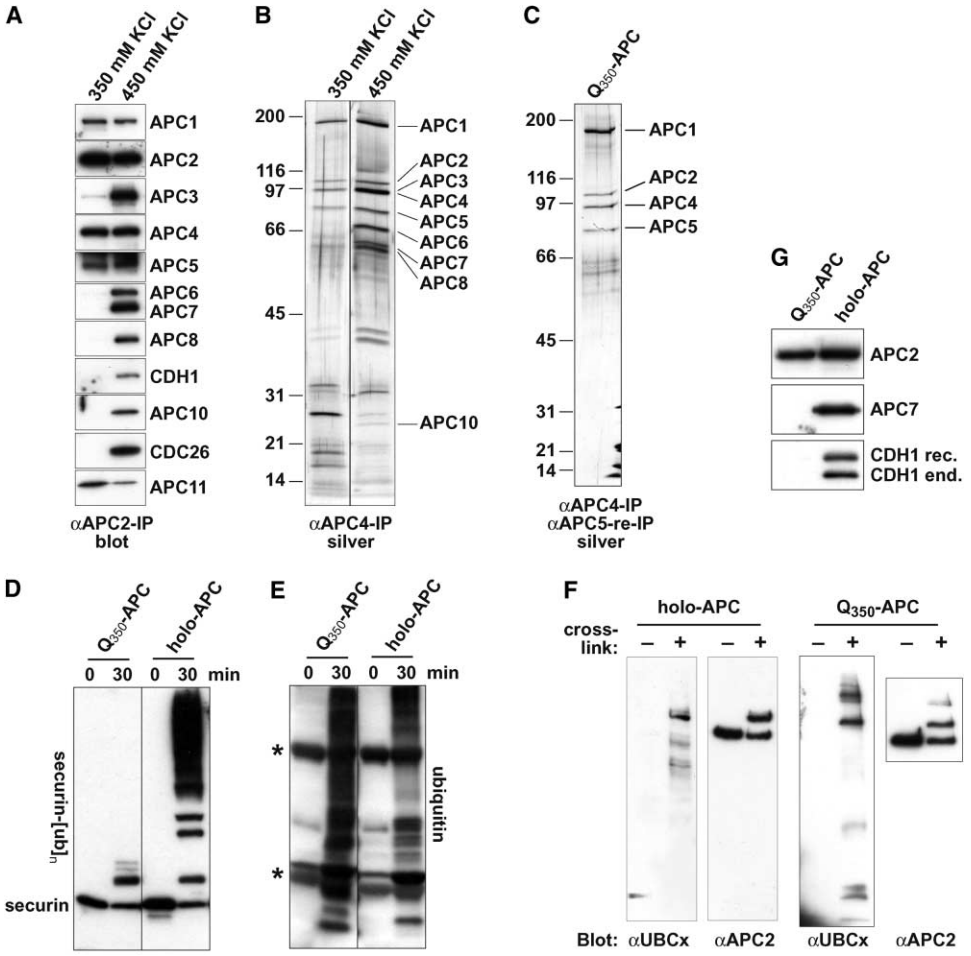

Figure 3. A Subcomplex Consisting of APC1, 2, 4, 5, and 11 Elutes Separately from Holo-APC from an Anion Exchange Resin

(A) αAPC2 IPs from the indicated elution fractions were separated by SDS-PAGE and were visualized by Western blotting.

(B) αAPC4 IP after predepletion of fraction 350 with αAPC3 antibodies. Samples were separated by SDS-PAGE and were visualized by silver staining.

(C) Silver staining of a parallel sample after peptide elution from the αAPC4 antibody beads and re-IP with αAPC5 antibodies.

(D) An in vitro ubiquitination assay with holo-APC or Q_{350}-APC and recombinant securin as substrate.

(E) The Western blot shown in (D) was reprobed with antibodies against ubiquitin. The asterisks denote crossreactions with the antibody used for IP.

(F) Crosslinking of UBCx to holo-APC (left) and Q_{350}-APC (right). Isolated complexes were incubated with modified UBCx and were analyzed by immunoblotting against UBCx and APC2.

(G) CDH1 binding assay. IPs of holo-APC or Q_{350}-APC were incubated with recombinant HisHA-tagged CDH1 and were immunoblotted for the presence of endogenous and recombinant CDH1.

APC2 in holo-APC as well as in Q_{350}-APC; this finding further corroborates the notion that Q_{350}-APC is not deficient in recruitment of E2 enzymes carrying activated ubiquitin. This implies that the deficiency to ubiquitinate securin might be a direct consequence of the absence of substrate specificity factors. Consistent with this hypothesis, we could not load recombinant CDH1 onto Q_{350}-APC, whereas APC from the 450 mM KCl fractions bound both endogenous and recombinant CDH1 (Figure 3G).

We conclude that Q_{350}-APC can interact with E2 enzymes and assemble multiubiquitin chains but cannot conjugate these chains to substrates. This deficiency is possibly due to a failure in substrate recruitment and may be a direct consequence of the absence of APC10 and the inability to interact with CDH1, two proteins implicated in substrate binding [12, 22].

The TPR Subunits APC3 and APC7 Bind the C Termini of CDH1 and CDC20

To understand APC activation, it is important to map the molecular interactions between the activators CDC20/CDH1 and APC subunits. Our analysis of Δ2/11-APC and Q_{350}-APC revealed that APC2 and APC11 are dispensable for CDH1 binding, and that APC1, APC2, APC4, APC5, and APC11 are not sufficient. This leaves CDC26, APC10, and the TPR proteins APC3, APC6, APC7, and APC8 as possible CDH1 receptors. Since multiple TPRs are present in several adaptor proteins like the cochaperone Hop/Sti1 or the peroxisomal tar-

geting signal receptor PEX5 [15], we inspected APC's TPR subunits closer by using a computational approach. Employing a heterogeneous selection of the best-performing structure prediction programs, the TPR domains in APC3, APC6, APC7, and APC8 were identified by a consensus as being closely related to the TPR region in PEX5 (see Table S1 in the Supplemental Data). Substrate binding of PEX5 involves an interaction of its TPRs with a conserved pentapeptide signal at the C terminus of the substrates [26, 27]. Using the Swiss-PDB viewer modeling environment [28], we threaded APC3's TPR region onto the backbone of the PEX5 structure (protein data bank id 1FCH, [26]) according to prediction, and we identified nine TPRs (instead of seven in PEX5) within residues 499–824. The four first and the four last TPRs are predicted to form two halves that are connected by TPR5 as a hinge (Figure 4A). This model suggested that TPR domains in the APC might form receptors for C-terminal peptide motifs. Consistent with this hypothesis, we have previously shown that APC3 interacts with APC10 through APC10's extended C terminus [9]. When we fitted APC10's C-terminal pentapeptide, YRSIR, into the structural model instead of PEX5's cognate PTS1 peptide (Figure 4A), we noted that this C-terminal motif, especially the ultimate IR residues, is conserved not only between APC10 orthologs from different species, but also among the APC activators of the CDC20/CDH1 family (Figure 4B). This remarkable conservation in several proteins in the APC context proposed an important, possibly common, functional role for this motif.

To test if APC3 interacts with the C termini of CDH1 and its related activator CDC20, we incubated insect cell lysates containing recombinantly expressed APC3 with synthetic peptides coupled to a matrix through their N terminus. About 10% of input APC3 bound to the C-terminal peptide of CDC20, and even stronger binding to a CDH1 C-terminal peptide was observed (Figure 5A). This interaction was specific, since APC3 did not bind to a control peptide, and recombinant APC5 interacted with neither the CDC20 nor the CDH1 peptide (Figure 5A). We next tested the other three TPR subunits and found that APC8 did not bind either peptide (Figure 5A). Likewise, binding of APC6 to the C-terminal peptides was weak and comparable to binding to a control peptide (Figure 5A). APC7, on the other hand, interacted strongly with the CDH1 C terminus and moderately with CDC20's C terminus (Figure 5A), in a manner very similar to APC3. We subsequently used APC7 to narrow down the region responsible for interaction. While an APC7 fragment containing the N-terminal 297 amino acids did not exhibit binding, the C-terminal part (amino acids 298–565) comprising the block of TPRs was strongly enriched on the peptide matrix (see Figure S3 in the Supplemental Data). These data indicate that the TPR domain is sufficient for interaction with IR tail peptides.

In order to exclude any nonspecific effects caused by incorrect folding of recombinantly expressed proteins, we searched for an alternative source of APC subunits in our peptide binding assays. As described above, late-eluting fractions from the Source 15Q anion exchange column contained considerable amounts of the TPR subunits APC3, APC6, and APC7, while most other sub-

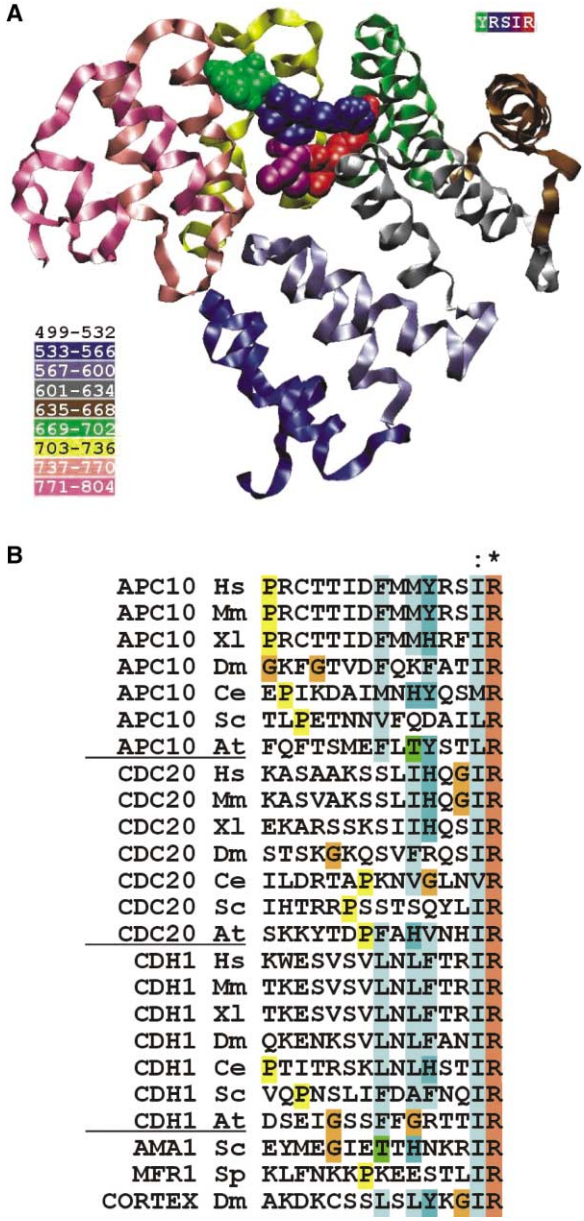

Figure 4. A Putative Interaction Module between TPR Subunits and APC Activators

(A) A three-dimensional model of the TPR domain of APC3, based on predicted structural homologies to PEX5. Individual TPRs are depicted in different colors. The PTS1 peptide crystallized with PEX5 has been replaced by the C-terminal pentapeptide of APC10 (YRSIR), which is known to interact with APC3.

(B) Alignment of the C termini of APC10, CDC20, and CDH1 orthologs, as well as the meiotic activators Ama1, Mfr1, and Cortex. The coloring of individual residues is based on physical propensities according to CLUSTAL_X. Organisms: *Homo sapiens*, Hs; *Mus musculus*, Mm; *Xenopus laevis*, Xl; *Drosophila melanogaster*, Dm; *Caenorhabditis elegans*, Ce; *Saccharomyces cerevisiae*, Sc; *Arabidopsis thaliana*, At; *Schizosaccharomyces pombe*, Sp. GenInfo numbers: 6463666, 20885472, 27505933 (EST, +2 translation), 28573741, 17559738, 6321197, 18398701; 4323528, 8885513, 3298595, 17137788, 17537825, 461700, 15240985; 7705377, 13879284, 2326943, 2326419, 17538129, 6321435, 22328875, 14486175, 26396380, 30172925.

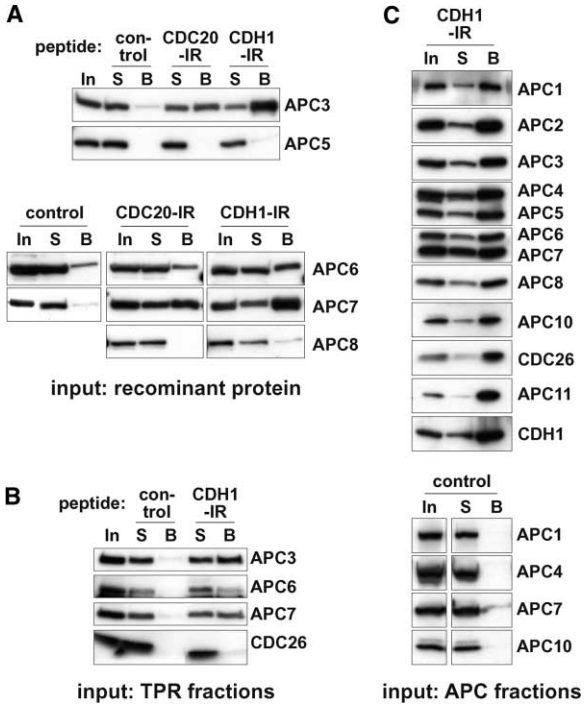

Figure 5. The TPR Subunits APC3 and APC7 Mediate APC Interactions with C-Terminal IR Motifs

(A) Matrix-coupled C-terminal peptides were incubated with insect cell lysates containing recombinant APC subunits. 10% of input (In), supernatant (S), and 100% of bound material (B) were analyzed by Western blot.

(B) Late-eluting Q column fractions (fractions 14–16) containing mainly TPR subunits were used as input in peptide binding assays.

(C) Fractions containing holo-APC were used as input in the described peptide binding assay.

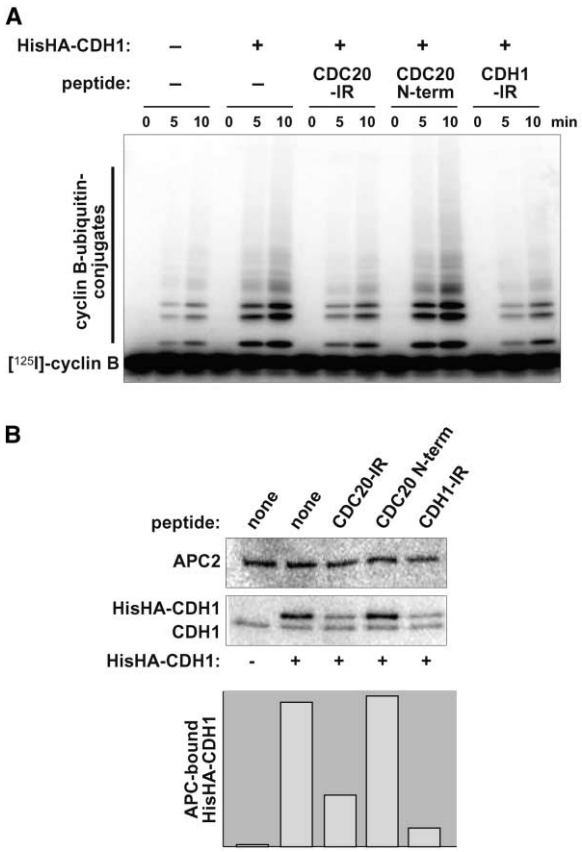

Figure 6. C-Terminal CDH1/CDC20 Peptides Containing the IR Motif Inhibit CDH1 Binding and APC Activation

(A) An in vitro ubiquitination assay. APC IPs were incubated with recombinant CDH1 in the presence or absence of different peptides, washed, and used in ubiquitination assays with [^{125}I]-labeled cyclin B as substrate.

(B) CDH1 binding assay. APC IPs treated as in (A) were analyzed by quantitative Western blotting with [^{125}I]-labeled secondary antibodies.

units were largely absent (Figure S2 and data not shown). Although these TPR subunits can still be partially coprecipitated, they do not seem to be part of stoichiometric complexes (data not shown). As seen in Figure 5B, endogenous APC6 bound only weakly, and the subunit CDC26, also present in these fractions, showed no binding at all. Conversely, APC3 and APC7 were again significantly retained by the CDH1 peptide matrix (Figure 5B), confirming the results obtained with recombinant proteins (Figure 5A). Interestingly, APC3 and APC7 are highly related in their primary sequences. In budding yeast, which lacks a gene for APC7, both proteins share Cdc27 as their closest homolog [29]. This suggests that APC3 and APC7 might have originated from gene duplication and might still have similar or overlapping functions.

Lastly, we wanted to know if CDH1's C terminus would be sufficient to interact with holo-APC. For this, we incubated APC-containing fractions with the peptide matrix. All subunits were strongly enriched on the CDH1 peptide matrix, but not on control peptides (Figure 5C). Since APC6 and CDC26 from late-eluting fractions did not bind the CDH1 peptide by themselves (Figure 5B), this indicated that holo-APC can bind to an IR motif peptide. Surprisingly, endogenous CDH1 was also enriched on the peptide matrix (Figure 5C). Since purified APC is loaded with substoichiometric amounts of endogenous CDH1 (Figures 1B and 3G), this cobinding could be mediated by dimerization of APC. Alternatively, more than one receptor for IR tails could be present per complex.

C-Terminal Peptides of CDH1 and CDC20 Inhibit APC Activation

Our results so far suggested that the interaction of IR tails in CDH1 and CDC20 with TPR subunits might facilitate recruitment of the activators to the APC. To test this hypothesis, we analyzed if IR tail peptides could compete for CDH1 binding to the APC and thereby prevent APC activation. Immunopurified APC was loaded with recombinant CDH1 in the presence or absence of IR tail or control peptides, washed, and used to ubiquitinate a radioactively labeled fragment of cyclin B. The C-terminal peptides of CDH1 and CDC20 both blocked APC activation efficiently, lowering ubiquitination activity to almost the level of APC that has not been activated by exogenous CDH1. In contrast, a peptide from the N-terminal region of CDC20 had no effect on APC activation (Figure 6A). Quantitative immunoblotting with anti-

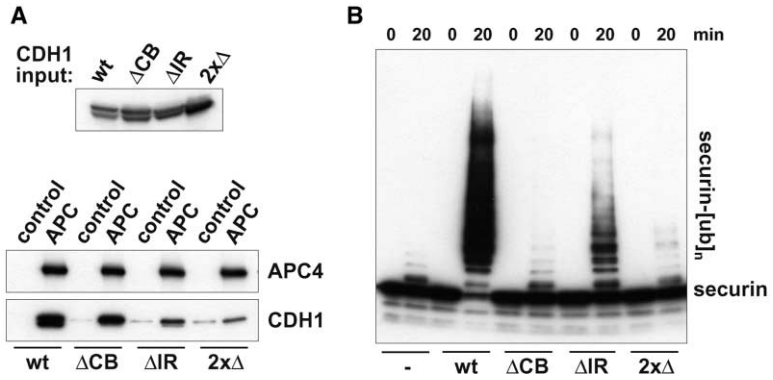

Figure 7. Binding and Activation of the APC Depends on CDH1's C-Terminal IR Motif and the C Box

(A) CDH1 binding assay. αAPC3 IPs from APC-containing fractions or APC-free control fractions were incubated with insect cell lysates containing recombinant wild-type CDH1 or CDH1 mutated in the C box (ΔCB), the IR motif (ΔIR), or in both motifs (2xΔ).

(B) An in vitro ubiquitination assay. APC was incubated with wild-type or mutant CDH1 as described above and was used in a ubiquitination assay with recombinant securin as substrate.

bodies against APC2 and CDH1 showed that the decreased activity corresponded with diminished binding of exogenous CDH1 (Figure 6B). As in the peptide binding assays (Figure 5), CDH1's C-terminal peptide had a stronger effect than the one from CDC20. The observed inhibition of CDH1 loading supports the notion that IR tail binding sites on the APC are required for CDH1 binding.

CDH1 Binding and APC Activation Depend on Two Motifs in CDH1

Having established that C-terminal IR tails are sufficient to interact with the APC and interfere with its activation, we wanted to know how their loss would influence CDH1 activity. To this end, we generated a baculovirus encoding CDH1 with a deletion of the C-terminal dipeptide (ΔIR). Another conserved motif located in CDH1's N-terminal region, the C box, has been shown to be required for APC binding in yeast [23]. We therefore also generated CDH1 versions with mutations in the C box (ΔCB) and in both motifs (2xΔ). After expression in insect cells, we incubated the lysates with antibody bound APC or control antibody beads, washed, and tested for binding of recombinant CDH1 and APC activation. Figure 7A shows that both mutations in the C box and in the IR tail diminished binding significantly but not completely. Combining both mutations resulted in a further decrease in binding, indicating that C box and IR tails cooperate to achieve optimal APC binding (Figure 7A).

Similar, but not identical, effects were seen in substrate ubiquitination assays. While the IR tail deletion mutant retained some activity, the C box mutant was almost completely inactive, even though it bound to APC in higher amounts (Figure 7B). Taken together, these data argue that both the C box and the IR tail are required for optimal CDH1 binding to the APC and APC activation.

Discussion

Despite the APC's essential role in progression through mitosis in presumably all eukaryotes, we still have a very limited understanding of the functions of its many subunits and about how they interact with the essential APC activator proteins CDC20 and CDH1. This lack of insight is partly due to the fact that it has not been possible to reconstitute the APC from recombinant subunits. We have thus searched for subcomplexes derived from native human APC and correlated their subunit composition with their functional properties. This reverse approach has yielded insights into both ubiquitination and substrate recognition by the APC.

Implications for the APC-Mediated Ubiquitination Reaction

The RING finger protein APC11 and its binding partner, the cullin homolog APC2, are able to assemble multiubiquitin chains ([4, 7, 8], Figure 2B). Our biochemical analysis of the Δ2/11-APC subcomplex has extended these observations by showing that APC2 and APC11 are not only sufficient for ubiquitination reactions in vitro, but are also strictly required for the activity of holo-APC (Figure 1C). In addition, crosslinking of UBCx indicates that APC2 is not only able to bind UBCx in vitro [8], but that it is also the only major UBCx-crosslinked subunit in the context of holo-APC and Q_{350}-APC (Figure 3F). Since APC11 and, to some extent, APC2/11 heterodimers are able to ubiquitinate APC substrates ([4, 7, 8], Figure 2B), whereas Q_{350}-APC subcomplexes are not (Figure 3D), it is possible that part of APC11's activity is due to an ability to freely interact with substrates, while the presence of additional subunits (e.g., APC1, APC4, APC5 in Q_{350}-APC) might restrain these interactions. Apparently, the competence of subcomplexes containing APC11 (APC2/11, Q_{350}-APC) to recruit E2 enzymes and assemble polyubiquitin chains is not sufficient for substrate ubiquitination. In the context of a larger APC assembly, substrate recruitment hence seems to strictly depend on the presence of specificity factors like CDH1 and CDC20.

TPR Subunits and APC Interactions with Substrate Specificity Factors

Binding of CDH1 to the APC is independent of the catalytic subunits APC2/APC11. On the other hand, the ability of CDH1 to bind to APC subcomplexes coincides with the presence of TPR subunits (Figures 1B and 3G). Moreover, peptides mimicking the C terminus of CDH1 and CDC20, which are essential for APC activation (see below), can bind to the TPR domain proteins APC3 and APC7 (Figures 5A and 5B). A role for these subunits in activator binding is further backed by a recent finding from budding yeast APC lacking the yeast-specific sub-

unit Apc9. This Δapc9-APC has reduced levels of Cdc27, the common closest yeast homolog of APC3 and APC7, and is compromised in its ability to bind Cdh1 [12].

The notion that APC3 and APC7 are receptors for CDH1's C terminus is supported by the prediction of structural homology to the peptide binding protein PEX5 and by the identification of a conserved motif, the IR tail, in the C terminus of CDH1 and CDC20 that is shared with APC10. Truncations of APC10's IR tail abolish binding to APC3 [9], and similarly, truncation of this motif in CDH1 strongly reduces CDH1's ability to bind and activate APC in vitro (Figure 7). Moreover, peptides containing this motif are sufficient to compete for CDH1 binding to the APC and can thereby prevent APC activation (Figure 6). The importance of the C terminus of CDH1 is bolstered by previous findings that C-terminally tagged budding yeast Cdh1 is nonfunctional and unable to interact with the APC [23, 30]. During preparation of this manuscript, Passmore et al. [12] furthermore reported that deletion or mutation of the IR tail in yeast Cdh1 or Cdc20 abolishes APC binding and activation in vitro.

While all these results support a role for the free IR tail in APC binding and activation, Jaspersen et al. [31] have shown that, in vitro, a C-terminal hexahistidine tag on yeast Cdh1 does not interfere with APC activation. A His tag may be too small to prevent the association of CDH1 with the APC, although this would argue against the type of interaction modeled in Figure 4A. Besides, Kallio et al. [32] reported that CDC20 tagged with the much larger GFP at the C terminus can coprecipitate with two APC subunits, although the functionality of this interaction has not been tested. It is therefore conceivable that the C box, another APC interaction motif previously identified in CDC20 and CDH1 ([23], Figure 7), was sufficient in these cases to allow APC association. Detailed structural studies will be required to analyze how exactly CDC20 and CDH1 interact with TPR subunits of the APC.

Interestingly, CDH1 and APC10 share the IR tail, are both only found in substrate recognition-competent subcomplexes containing TPR subunits, and have both been implicated in substrate binding [12, 22]. It is therefore conceivable that CDH1 first recruits substrates to the APC, whereas APC10 helps to sequester substrates during the elongation of ubiquitin chains [11, 12]. The DOC domain in APC10, which is also found in other ubiquitin ligases [33–35] and is likely to coordinate an unidentified ligand [9, 10], may have an important role in this substrate binding and positioning reaction.

Is Modularity the Reason for the APC's Complexity?

Both CDC20/CDH1 and APC10 appear to bind APC through the same conserved C-terminal motif, and endogenous CDH1 is furthermore found associated with APC bound to an IR peptide (Figure 5C). This makes it likely that there are multiple binding sites for IR tails. Our data are consistent with this possibility, since both APC3 and APC7 interact with IR peptides in vitro (Figures 5A and 5B). In addition, each TPR subunit may be present in more than one copy within holo-APC ([36], C.G. and H.C.V., unpublished data). A computational search ("Evoluation," see the Supplemental Data for a brief description) for proteins with C-terminal IR motifs identified several more candidates, among them the ubiquitin-activating enzyme E1 and the cell-cycle phosphatase CDC14B.

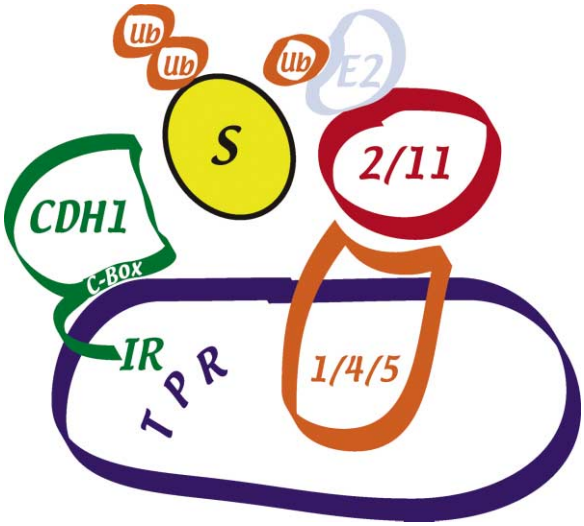

Figure 8. Cartoon Summarizing Our Current Views of APC Assembly and Mechanism

APC2/11 recruit ubiquitin-loaded E2, and they can be dissociated from the complex. They can also form a stable complex together with APC1, 4, and 5 in the absence of other subunits, especially the TPR proteins. CDH1 interacts with the TPR subunits APC3 and APC7 and might thereby be juxtaposed to APC2/11. This model is consistent with CDH1's proposed role as a substrate-presenting factor.

TPRs are the most common sequence motifs identified in APC subunits so far, being present in contiguous blocks of six or more repeats in at least four subunits of the vertebrate APC. In yeast, all TPR subunits are essential for viability, and mutations in their genes have frequently been picked up in screens for temperature-sensitive cell division cycle (*cdc*) mutants, underscoring the important role of these proteins in mitotic progression [13, 18, 37–41]. Nonetheless, apart from the notion of a general role in mediating protein-protein interactions, their precise functions within the APC had remained enigmatic. For two of these subunits, APC3 and ACP7, our observations now indicate an important role in activator binding through an extended peptide. Notably, two amino acid exchanges in described APC3-TPR domain mutants of different species [42, 43] are predicted by our structural model to be in close proximity of the C-terminal arginine residue of the IR peptide (data not shown).

TPR domain interactions with C-terminal peptide motifs have been described for the peroxisomal targeting signal PTS1 and its receptor PEX5 [15, 26], and for the C termini of Hsp70/Hsp90 and several interacting TPR proteins like Hop [44, 45]. APC3 and APC7 appear to represent a third class of TPR proteins that bind interacting proteins via their C termini. Since the predicted

structures of APC6 and APC8 also match PEX5 (Table S1), it is tempting to speculate that they too might share this property. In this way, the TPR subunits could serve as versatile adaptor sites for a variety of interacting proteins and thus rationalize some of the APC's complexity.

Conclusions

Our isolation and characterization of two subcomplexes of the human APC have provided first insight into the molecular interactions between APC's many subunits (Figure 8). The cullin subunit APC2 and its binding partner, the RING finger protein APC11, are found in a subcomplex with APC1, APC4, and APC5 and are essential for the assembly of multiubiquitin chains from ubiquitin residues donated by E2 enzymes. Substrate ubiquitination requires the activator proteins CDH1 and CDC20, which interact via their C termini with the TPR subunits APC3 and APC7. APC's TPR subunits are predicted to form structures that are similar to the one of the peroxisomal import receptor PEX5, which binds cargo proteins via their C termini. The APC may therefore contain multiple TPR subunits to allow modular interactions with different regulatory proteins. These results reveal a function for the TPR subunits of the APC, and they provide insight into how substrates are recruited to the ubiquitin ligase.

Supplemental Data

Supplemental Data including three additional figures, a summary of computational approaches, and the Experimental Procedures are available at http://www.current-biology.com/cgi/content/full/13/17/1459/DC1/.

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