The WD40 Propeller Domain of Cdh1 Functions as a Destruction Box Receptor for APC/C Substrates

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Summary

Activation of the anaphase-promoting complex/cyclosome (APC/C) by Cdc20 and Cdh1 leads to ubiquitin-dependent degradation of securin and cyclin B and thereby promotes the initiation of anaphase and exit from mitosis. Cyclin B and securin ubiquitination depend on a destruction box (D box) sequence in these proteins, but how APC/C bound to Cdc20 or Cdh1 recognizes the D box is poorly understood. By using site-specific photocrosslinking in combination with mutational analyses, we show that the D box directly interacts with an evolutionarily conserved surface on the predicted WD40 propeller structure of Cdh1 and that this interaction is essential for processive substrate ubiquitination. We further show that Cdh1 specifically crosslinks to the APC/C subunit Cdc27 and that Cdh1 binding to APC/C depends on the presence of Cdc27. Our data imply that APC/C is activated by the association of Cdh1 with Cdc27, which enables APC/C to recognize the D box of substrates via Cdh1’s propeller domain.

Introduction

Chromosome segregation and subsequent exit from mitosis or meiosis depends on the degradation of A and B type cyclins, the activating subunits of cyclin-dependent kinase 1 (Cdk1), and on the destruction of securin, an inhibitor of the protease separase, which initiates anaphase by cleaving chromosome cohesion proteins. The degradation of cyclins, securin, and several other mitotic proteins is initiated by a 1.5 MDa ubiquitin ligase complex called the anaphase-promoting complex or cyclosome (APC/C) that assembles multibuquitin chains on substrates and thereby targets them for destruction by the 26S proteasome (reviewed by Peters [2002]).

The activity of APC/C is strictly controlled by the regulated binding of one of several activating proteins. In mitosis, the activator Cdc20 associates with APC/C once several APC/C subunits have been phosphorylated (Shteynberg et al., 1999; Kramer et al., 2000; Rudner and Murray, 2000; Kraft et al., 2003), and the resulting APC/C-Cdc20 holo complex initiates securin and cyclin B degradation when all chromosomes have been aligned on the spindle in metaphase. Once cyclin B destruction has reduced Cdk1 activity, mitotic phosphate groups are removed by phosphatases from the activator Cdh1/Hct1, which allows this protein to associate with APC/C, target Cdc20 for destruction, and keep APC/C active until the end of G1 phase when Cdh1 becomes rephosphorylated (Zachariae et al., 1999; Kramer et al., 2000). Cdc20 and Cdh1 are characterized by a “C box” sequence in the N-terminal domain and an “IR tail” at the very C terminus, both of which are required for APC/C binding (Schwab et al., 2001; Passmore et al., 2003; Vodermaier et al., 2003), and by the presence of a C-terminal WD40 domain that is predicted to fold into a seven-bladed β-propeller structure.

The ubiquitination of APC/C substrates depends on the presence of at least one of several specific sequence elements in cis. The best characterized of these is the destruction box (D box; Glotzer et al., 1991), which is found in the N termini of A and B type cyclins and of securin, but several APC/C substrates contain, either instead or in addition, a KEN box (Pfleger and Kirschner, 2000). How these sequence elements contribute to substrate ubiquitination and how substrates are recognized by APC/C is not well understood. In budding yeast, overexpression of Cdc20 and Cdh1 activates APC/C in a substrate-specific manner, and deletion of the nonessential CDH1 gene stabilizes only some but not all APC/C substrates (Schwab et al., 1997; Visintin et al., 1997), raising the possibility that Cdc20 and Cdh1 function as substrate adaptors (Vodermaier, 2001), as do some other WD40 proteins in the case of the ubiquitin ligase SCF (Orlicky et al., 2003; Wu et al., 2003). Consistent with this possibility, it has been shown that substrates can only associate with APC/C in the presence of Cdc20 or Cdh1 (Passmore et al., 2003) and that Cdc20 and Cdh1 can bind to APC/C substrates directly (Ohtoshi et al., 2000; Burton and Solomon, 2001; Hilioti et al., 2001; Pfleger et al., 2001; Schwab et al., 2001; Sorensen et al., 2001), although there is no consensus as to whether binding occurs to the N termini (Pfleger et al., 2001) or to the WD40 domains of Cdc20 and Cdh1 (Hilioti et al., 2001; Sorensen et al., 2001). Furthermore, only some of these interactions were found to depend on D or KEN boxes, and there is only little evidence so far that the observed binding of APC/C substrates to Cdc20 and Cdh1 is actually required for substrate ubiquitination (Sorensen et al., 2001).

The hypothesis that Cdc20 and Cdh1 function as substrate adaptors has recently been challenged by the observation that a tandemly repeated N-terminal fragment of fission yeast cyclin B associates with a D box-dependent manner with APC/C but not with Cdc20 in mitotic Xenopus egg extracts (Cdh1 is not present in Xenopus eggs and could therefore not be analyzed in these experiments [Yamano et al., 2004]). It is unknown to which of APC/C’s twelve known subunits cyclin B bound under these conditions, but the Doc1/Apc10 protein is an attractive candidate. The structure of this protein suggests a ligand binding function...
Characterization of Crosslinker Probes for the Identification of D box Receptors on APC/C

To analyze which APC/C subunits or activators interact with the D box of APC/C substrates we generated synthetic peptides that represent parts of human cyclin B1 (amino acid residues 36–64; "CB") and human securin (residues 55–86; "SE"), in which the photoactivatable amino acid benzophenone-phenylalanine (BPA) was incorporated in direct proximity to the D box (Figures 1A and 2A). For specificity controls, we synthesized peptides in which the order of amino acid residues was randomly changed (scrambled, sc) and peptides in which the conserved arginine and leucine residues of the D boxes were mutated to alanine (D box mutants, dm).

Before using these molecules as crosslinking probes, we asked if and how specifically the peptides are recognized by APC/C. At a concentration of 300 μM, the D box-containing peptides, but not their scrambled or D box mutant counterparts, strongly inhibited the cyclin B ubiquitination activity of purified Xenopus interphase APC/C, which had been activated with recombinant purified Cdh1 (Figure 1B and data not shown). Peptides containing BPA had the same effect in this assay as peptides without BPA (Figure 1B). When biotinylated peptides were used as substrates in reaction mixtures containing APC/C(Cdh1), cyclin B and securin peptides were ubiquitinated, whereas the scrambled and D box mutant peptides were not (Figure 1C). These results demonstrate that D box peptides can be recognized as substrates by APC/C(Cdh1). Their recognition by APC/C(Cdh1) does not depend on their susceptibility to ubiquitination because cyclin B peptides that are lacking all lysine residues could inhibit APC/C(Cdh1) as efficiently as peptides containing lysine residues (data not shown).

In agreement with earlier reports (Yamano et al., 1998; Waizenegger et al., 2000), we observed that both peptides delayed the degradation of cyclin B in Xenopus egg extracts in a D box-dependent manner, although the peptides did not prevent progression of these extracts to a mitotic state where APC/C(Cdc20) is active (mitotic entry was monitored by analyzing an electrophoretic mobility shift of Cdc25C; Figure 1D). These observations indicate that our synthetic peptides are recognized by APC/C in a similar way as natural substrates.

Cyclin B and Securin Crosslink to Cdc20 and Cdh1 in a D Box-Dependent Manner

To identify D box receptors, we initially tried unbiased approaches in which we searched for proteins in mitotic Xenopus extracts that could be crosslinked to cyclin B peptides. Although a number of crosslink products could be detected, none of them was D box dependent (data not shown), indicating that cyclin B
peptides can interact with a number of proteins, presumably in a nonspecific manner. We therefore did not analyze these interactions further.

Next, we asked if cyclin B peptides can be crosslinked to APC/C and/or Cdc20 in mitotic *Xenopus* extracts. Biotinylated BPA peptides were incubated in *Xenopus* extracts, crosslinking reactions were activated by exposure to UV light, and peptides and crosslinked molecules were then isolated on an avidin matrix and analyzed by SDS-PAGE and immunoblotting with APC/C and Cdc20 antibodies. Lanes 4–6 in Figure 2B show that similar amounts of the APC/C subunit Cdc27 could be detected in the resulting fractions, independent of whether wild-type cyclin B or the corresponding scrambled or D box mutant peptides had been used, indicating that the BPA peptides crosslink to some subunit of APC/C in a sequence independent manner.

In contrast, Cdc20 could only be detected in fractions obtained with wild-type cyclin B peptide, suggesting that binding of cyclin B to Cdc20 or to some Cdc20-associated protein depends on the D box (Figure 2B). In the same experimental setup, Cdc20 could also be detected when crosslinking reactions were performed in APC/C-depleted extracts (Figure 2B, lanes 1–3), but under these conditions much less Cdc20 was detected in cyclin B crosslink fractions than in the presence of APC/C (Figure 2B, compare lanes 1 and 4), implying that cyclin B peptides interact more efficiently with Cdc20 when it is bound to APC/C. Also, securn peptide crosslinking to Cdc20 in a D box-dependent manner (Figure 2D), suggesting that this interaction might be common to all D box-containing APC/C substrates.

Next, we analyzed if cyclin B D box peptides bind directly or indirectly to Cdc20. Following crosslinking, APC/C was immunoprecipitated from *Xenopus* extracts, the immunoprecipitates were denatured with SDS, and cyclin B peptide-associated molecules were isolated on an avidin matrix. Under these conditions, only proteins that are directly crosslinked to the biotinylated peptides are recovered. SDS-PAGE and immunoblotting revealed that Cdc20 was also isolated in a D box-dependent manner by this procedure, indicating that cyclin B D box peptides directly interact with Cdc20 (Figure 2C).

To confirm and extend this hypothesis, we analyzed if D box peptides also specifically crosslink to purified recombinant Cdc20 and Cdh1. After UV treatment, Cdc20 and Cdh1 were immunoprecipitated, separated by SDS-PAGE, and crosslinked peptides were visualized by biotin Western blotting. Wild-type peptides strongly crosslinked to Cdc20 and Cdh1, whereas D box mutant and scrambled peptides were crosslinked less efficiently (Figure 2E). These differences were not due to a general ability of wild-type peptides to crosslink more efficiently than control peptides because all three peptides could be crosslinked very weakly but with similar efficiencies to an unrelated control protein (a fragment of the condensin subunit Smc2; data not shown). In the same assay, peptide crosslinking to Cdh1 did not depend on Cdh1’s IR tail or an intact C box (Figure 2F). Cyclin B peptides can therefore interact directly with Cdc20 and Cdh1 in a D box-dependent manner.

In similar experiments, we observed that cyclin B peptides could be crosslinked to several subunits of purified APC/C, but none of these interactions was sequence specific (data not shown). We therefore did not characterize these interactions further.

An Evolutionarily Conserved Surface on One Side of Cdh1’s WD40 Propeller Is Required for Substrate Binding and Processive Substrate Ubiquitination

Our observations so far were consistent with the possibility that Cdc20 and Cdh1 function as substrate adaptors. An important prediction of this hypothesis is that substrate binding to the activator proteins should be required for substrate ubiquitination by APC/C. To test this prediction, we searched for activator mutants that are defective in substrate binding and analyzed their ability to activate APC/C. We chose Cdh1 for these experiments because APC/C activation by Cdh1 does not depend on mitotic APC/C phosphorylation, unlike activation by Cdc20 (Kramer et al., 2000; Kraft et al., 2003), and is therefore easier to reconstitute with purified components. We hypothesized that if the observed in-
Figure 3. Mutations on Cdh1’s WD40 Propeller Decrease Substrate Binding and APC/C Processivity

(A) Top and bottom views of models of the WD40 propellers of Cdc20 and Cdh1. Red signifies highest, white signifies intermediate, and green signifies lowest evolutionary conservation. Residues analyzed by alanine mutagenesis are indicated. Yellow arrows mark residues mutated in D12, and blue arrows mark (tmd)phe integration sites.

(B) Table of Cdh1 mutants and their properties. In APC/C binding assays wild-type Cdh1 was set to 100%; n.a., not analyzed.

(C) In vitro translated 35S-labeled wild-type (wt) or mutated Cdh1 or an unrelated WD40 protein (rat PP2A subunit PR55/Bα) were incubated with cyclin B peptides, exposed to UV light and isolated on avidin resin. Bound proteins were analyzed by SDS-PAGE/phosphorimaging, and signal intensities were quantified.

(D) Comparison of cyclin B wild-type (wt) and D box mutant (dm) peptides in crosslink assays as in (C).

(E) Binding of in vitro translated 35S-labeled wild-type or mutated Cdh1 to purified APC/C; IR, IR tail deleted.

(F and G) Xenopus interphase APC/C was loaded with comparable amounts of in vitro translated 35S-labeled wild-type or mutated Cdh1 (F) and APC/C activity was measured at different time points using 125I-labeled cyclin B fragment (aa 1–87) as substrate (G).

Interactions between substrates and activators were important for APC/C function, they should be evolutionarily conserved. We had already observed that the conserved C box and IR tail of Cdh1 are not required for substrate crosslinking (Figure 2F). We therefore analyzed another evolutionarily conserved sequence in Cdh1, the WD40 domain. This domain is predicted to form a β-propeller structure, which in other proteins is known to mediate protein-protein interactions.

We measured the conservation of residues in multiple alignments of Cdc20 and Cdh1 sequences from a taxonomically wide-spread selection of organisms, modeled the WD40 domains of these proteins, and mapped the degree of conservation onto the molecular surface of the predicted structures (Figure 3A; red, white, and green represent high, medium, and low conservation, respectively; see Supplemental Data available with this article online). For Cdh1 we observed that 24 highly conserved (red) residues were clustered on one side of the disk-shaped propeller domain, whereas the opposite side appeared less well conserved (Figures 3A and S1). We tentatively refer to the side with more conserved residues as the “top side” and to the other one as the “bottom side.” Cdc20’s propeller domain shows a similar distribution of conserved residues between the top and the bottom side, although the degree of conservation on the top side is not as pronounced as it is in the case of Cdh1.

We “scanned” the top surface of Cdh1’s propeller domain by mutating 17 out of its 24 highly conserved residues (Figures 3A, 3B, and S1). To avoid disruption of the propeller structure, we only mutated residues that are predicted to lie in loop regions between the β-sheets of the WD40 propeller. The resulting mutants were synthesized as 35S-labeled proteins by in vitro transcription and were analyzed for their ability to be crosslinked to cyclin B D box peptides. The peptide-associated fraction was isolated on an avidin matrix and the amounts of bound Cdh1 proteins were visualized by phosphorimaging and quantified. Figure 3C shows that some mutations did not alter the cross-linking efficiency of Cdh1 to cyclin B peptides (A2), whereas others reduced the crosslinking efficiency either partly (D2), or in one case strongly (D12). In the
latter D12 mutant four conserved amino acid residues that are clustered in one area of the top surface of the propeller domain have been changed simultaneously (L179V, P182A, G463A, D464A; Figures 3A and 3B). This mutant could only be crosslinked to cyclin B peptides in small amounts that are comparable to the amounts of an unrelated WD40 protein (a PP2A subunit) that were crosslinked to the same peptides (Figure 3C) and to the low amounts of wild-type Cdh1 that can be crosslinked to the D box mutant peptide (Figure 3D), indicating that these interactions are nonspecific.

Next, we analyzed if the Cdh1 mutants are able to bind and activate APC/C. Unexpectedly, we found that the ability of all mutants to bind APC/C was somewhat reduced (Figure 3B). In the case of the D12 mutant, 2-fold less Cdh1 bound to APC/C under our test conditions than wild-type Cdh1 (Figure 3B). However, the binding of D12 to APC/C was still dependent on Cdh1’s IR tail (Figure 3E), arguing against the possibility that this mutant is simply misfolded and therefore binds nonspecifically to APC/C. These observations raise the possibility that the residues that are mutated in D12 are directly interacting with APC/C. However, since the same residues are also required for efficient substrate binding (Figure 3C), it is also possible that the ability of this surface to bind substrates indirectly alters the efficiency of Cdh1 binding to APC/C.

To be able to distinguish between effects on APC/C activity that are caused by reduced APC/C binding, and by effects that are caused by reduced substrate binding, we titrated the amounts of Cdh1 proteins that were added to APC/C so that similar amounts of wild-type and mutant proteins were loaded onto APC/C (Figure 3F). We then analyzed the ability of the resulting forms of APC/C<sub>Cdh1</sub> to ubiquitinate a radiolabeled fragment of cyclin B. In all cases, we found that the ability of Cdh1 mutants to bind substrates (Figure 3C) correlated with their ability to activate APC/C (Figures 3B and 3G). The D12 mutant, which showed the biggest reduction in substrate binding, also showed the biggest difference in APC/C activation assays compared to wild-type Cdh1. Interestingly, APC/C bound to D12 was specifically defective in generating high molecular mass cyclin B-ubiquitin conjugates but instead generated more low molecular mass conjugates in which between one and four ubiquitin residues were bound to one cyclin B molecule (Figure 3G). The same four residues that are mutated in D12 are therefore required for efficient substrate binding and for the ability of APC/C<sub>Cdh1</sub> to ubiquitinate substrates in a processive manner. This correlation strongly supports the hypothesis that substrate binding to Cdh1 is required for substrate ubiquitination by APC/C.

Cdh1 Specifically Crosslinks to the Cdc27 Subunit of APC/C

If Cdc20 and Cdh1 function as D box receptors, then it will be important to understand how Cdc20 and Cdh1 are recruited to APC/C to present substrates there. We and Passmore et al. (2003) showed previously that Cdh1’s IR tail is required for APC/C binding, and we found that peptides containing this tail can bind to recombinant forms of the APC/C subunits Cdc27/Apc3 and Apc7 (Vodermaier et al., 2003). However, it is not known if full-length Cdh1 binds to these subunits and if it also does so in the context of APC/C.

To address this question in an unbiased manner, we developed a crosslinking strategy similar to the one we had used for mapping APC/C-substrate interactions. Because we wanted to use properly folded full-length Cdh1, we could not use short synthetic peptides as crosslinking probes. Therefore, we introduced the photoactivatable amino acid 3-trifluoromethyl-3-phenyl-diazirine ([tmd]phe) cotranslationally into Cdh1, using the approach developed by Brunner [1993]. We introduced amber stop codons at different positions in the Cdh1 cDNA, transcribed the resulting mutated cDNAs in vitro, and performed translation reactions in the presence of an amber stop codon suppressor tRNA which had been coupled to (tmd)phe. Under these conditions, translation beyond the amber codon only proceeds if the suppressor tRNA has incorporated (tmd)phe into the nascent polypeptide chain. Each fully translated protein therefore contains the photoactivatable amino acid and can participate in crosslinking reactions. Figure 4A shows the in vitro translation products of a Cdh1 mutant in which phenylalanine codon 444 was changed to an amber stop codon. In the absence of suppressor (tmd)phe-tRNA, only truncated Cdh1 was generated, whereas in the presence of suppressor tRNA, about half of the translation products represented full-length protein that had integrated (tmd)phe. The incorporation of (tmd)phe into Cdh1 did not reduce its ability to activate APC/C (data not shown).

The (tmd)phe residue was integrated at five different sites in Cdh1, in direct proximity to either the C box (serine 51) or the IR tail (phenylalanine 489), or in areas that are predicted to be on the top (serine 400, phenylalanine 444) or bottom side of the WD40 propeller (arginine 240; Figures 3A and 4B). The resulting radiolabeled (tmd)phe-containing Cdh1 proteins were incubated with immunopurified HeLa APC/C and crosslinking reactions were activated with UV light. The samples were then denatured with SDS and subjected to immunoprecipitation with APC/C subunit-specific antibodies. For subunits Apc2, Cdc27, Apc4, Apc5, Cdc16, and Apc7 (which range in molecular mass from 105–62 kDa) we characterized antibodies that are able to selectively immunoprecipitate their corresponding antigen under SDS denaturing conditions (Figure 4C and data not shown). We did not characterize antibodies with the same properties for the other six known APC/C subunits because we only obtained Cdh1/APC/C crosslink products whose mass is inconsistent with them containing either APC1 (200 kDa) or one of the lower molecular mass APC/C subunits (see below).

All Cdh1 mutants except S400 amber yielded crosslink products with masses of 150–200 kDa whose generation was dependent on the presence of APC/C, exposure to UV light and Cdh1’s IR tail (Figure 4B, D). In all four cases, the APC/C-dependent crosslink products could only be immunoprecipitated with either one of two different antibodies specific for Cdc27, but not with any of the other five antibodies tested (Figure 4B and data not shown). In immunoprecipitation experiments Cdc27 antibodies are highly specific for APC/C as judged by mass spectrometric analysis and by SDS-PAGE and silver staining (Kraft et al., 2003). Cdh1
Figure 4. Cdh1 Specifically Crosslinks to Cdc27
(A) Phosphorimage showing in vitro translated 35S-labeled wild-type (wt) and mutated (Y444amber) Cdh1 in the presence or absence of (tmd)phe suppressor tRNA.
(B) Top panel: structure of (tmd)phe. Middle panels: HeLa log-phase APC/C was incubated with (tmd)phe-containing Cdh1 mutants, exposed to UV light, denatured with SDS, and immunoprecipitated using APC/C subunit-specific antibodies. Immunoprecipitates and aliquots of the total reaction mix (tot) were analyzed by SDS-PAGE/phosphorimaging. Bottom panel: bar diagram of Cdh1 showing the positions of C-box (black), WD40 domain (light gray), and amber mutations (stars).
(C) Comparison of APC/C antibodies in native (N) and denaturing (D) immunoprecipitations. Proteins immunoprecipitated from interphase HeLa cell extracts were eluted from antibody beads with low pH and analyzed by immunoblotting.
(D) Crosslink analysis of Cdh1-Y444JIR as in (B).
(E) Top panel: structures of SMCC, BMOE, and BPM. Bottom panels: in vitro translated 35S-labeled wild-type Cdh1 was loaded onto interphase HeLa APC/C bound to antibody-beads, incubated with SMCC, BMOE, BPM, or DMSO, in the case of BPM exposed to UV light, denatured with SDS, immunoprecipitated with APC/C antibodies, and analyzed by SDS-PAGE/phosphorimaging.

Crosslink products obtained with Xenopus APC/C could also be immunoprecipitated with Cdc27 antibodies (data not shown), but not with Apc7 antibodies (antibodies to other Xenopus APC/C subunits were not available), indicating that Cdh1’s predominant binding partner in APC/C is Cdc27.

To confirm the results obtained with (tmd)phe-containing Cdh1, we also used other chemical crosslinkers to analyze with which APC/C subunit Cdh1 interacts. Immunopurified HeLa APC/C was incubated with in vitro-translated Cdh1 followed by treatment with the crosslinking reagents SMCC, a lysine-cysteine-specific heterobifunctional crosslinker, BMOE, which reacts with two cysteine side chains, or BPM, which reacts with cysteines and upon UV activation with C-H bonds. Although many more crosslink products were obtained than in experiments using (tmd)phe-containing Cdh1, denaturing immunoprecipitation experiments clearly confirmed that Cdh1 was specifically crosslinked to Cdc27 (Figure 4E).

We also generated five (tmd)phe-containing forms of Cdc20. Unfortunately, none of these yielded discrete crosslink products with mitotic APC/C subunits that could be identified. The reason for this discrepancy between Cdh1 and Cdc20 behavior is unknown, but it is possible that a higher proportion of in vitro translated Cdc20 molecules is misfolded and thus binds to APC/C in a nonspecific fashion. It is also possible that Cdc20 can be crosslinked to APC/C less well due to the requirement for mitotic APC/C phosphorylation in this reaction.

Cdc27 Is Required for the Association of Cdh1 and Cdc20 with Budding Yeast APC/C
To test the hypothesis that Cdc27 represents the Cdh1 receptor on APC/C, we analyzed the ability of Cdh1 to associate with forms of APC/C that are lacking Cdc27. Since such APC/C forms cannot easily be generated in vertebrate cells, we performed these experiments in budding yeast. In S. cerevisiae, the CDC27 gene is essential for viability, but this requirement can be overcome by introducing multiple copies of the SIC1 gene under its own cell cycle regulated promoter. The resulting strains express elevated levels of the Cdk1 inhibitor Sic1 at the end of mitosis and are therefore able to exit mitosis despite compromised cyclin proteolysis (Thompson and Toczyski, 2003). We created such Jcdc27 “bypass suppression” strains in which Apc4 was fused to
a C-terminal tandem affinity purification (TAP) tag to allow isolation of APC/C. In the same strains, Apc5 was fused to three C-terminal haemagglutinin (HA) tags and Cdh1 to an N-terminal myc tag to facilitate their detection.

We purified APC/C from \( \Delta cdc27 \) and isogenic CDC27 strains using the TAP tag and compared the subunit composition of both forms of APC/C by SDS-PAGE, silver staining, immunoblotting, and mass spectrometry. As predicted, Cdc27 could not be detected in APC/C isolated from \( \Delta cdc27 \) strains. In addition, Cdc16 and Cdc26 were also reduced in their abundance. All other subunits were present in normal quantities (Figures 5A and 5B and data not shown). To test the integrity of \( \Delta cdc27 \)-APC/C, we performed sucrose density gradient centrifugation, which revealed that APC/C purified from \( \Delta cdc27 \) strains still sedimented as a high molecular mass particle, although its sedimentation coefficient was slightly reduced compared to that of wild-type APC/C (Figure 5C). It has previously been shown that APC/C in total yeast lysates sediments with a sedimentation coefficient of 36S (Zachariae et al., 1996), but we found that purified yeast APC/C has a sedimentation coefficient of 22S, identical to that of human and frog APC/C (Figure 5C). As dimeric forms of yeast APC/C have been observed previously (Passmore et al., 2003), it is possible that yeast APC/C oligomerizes or binds to other protein complexes in crude extracts and in vivo.

We could not detect endogenous Cdh1 in purified APC/C samples, even when APC/C was isolated from wild-type strains, presumably due to the low levels of Cdh1 in yeast (Zachariae et al., 1998). We therefore analyzed if recombinant yeast Cdh1 generated by in vitro translation can bind equally well to wild-type and \( \Delta cdc27 \) APC/C in vitro. TAP-tagged APC/C bound to IgG sepharose, incubated with in vitro translated \(^{35}S\)-labeled yeast Cdh1, Cdh1\( \Delta IR \), Cdc20 and Cdc20\( \Delta IR \), and bound material was eluted by TEV cleavage and analyzed by SDS-PAGE/phosphorimaging.

Figure 5. Cdc27 Is Required for the Association of Cdc20 and Cdh1 with Yeast APC/C

(A and B) Tandem affinity purification (TAP) of APC/C from CDC27 and \( \Delta cdc27 \) strains. EGTA-eluted samples were analyzed by SDS-PAGE and silver staining (A) or HA and Cdc27 immunoblotting (B).

(C) TAP-purified CDC27 and \( \Delta cdc27 \) yeast APC/C, and APC/C immunopurified from interphase HeLa cells and interphase Xenopus egg extracts, were analyzed by 10%–40% sucrose density gradient centrifugation and immunoblotting with the indicated antibodies.

(D) TAP-tagged yeast APC/C was bound to IgG sepharose, incubated with in vitro translated \(^{35}S\)-labeled yeast Cdh1, Cdh1\( \Delta IR \), Cdc20 and Cdc20\( \Delta IR \), and bound material was eluted by TEV cleavage and analyzed by SDS-PAGE/phosphorimaging.

\( \text{Cdh1's IR Tail Is Essential for Viability in Budding Yeast} \)

The binding of Cdh1 to APC/C in vitro depends on Cdh1's IR tail (Passmore et al., 2003; Vodermaier et al., 2003; Figures 3E, 3F, and 5D), but it has never been tested if this short sequence element is required for Cdh1 function in vivo. We therefore analyzed the physiological importance of the IR tail in budding yeast.
Cdh1 is not essential for viability in yeast unless the SIC1 gene is deleted (Schwab et al., 1997; Visintin et al., 1997), indicating that cyclin proteolysis mediated by Cdh1, and Cdk1 inhibition by Sic1, function redundantly. We therefore generated diploid yeast strains in which one SIC1 allele was deleted and in which one CDH1 allele was replaced by CDH1ΔIR. After sporulation, we tested by tetrad analyses if haploid sic1Δcdh1ΔIR cells could be obtained. This was never the case (data not shown), indicating that Cdh1’s IR tail is essential for the function of Cdh1 in vivo. This observation also strengthens the notion that the IR tail-dependent binding of Cdh1 to APC/C in vitro reflects a physiologically relevant reaction.

Discussion

The ability of APC/C to ubiquitinate mitotic cyclins, securin, and other D box-containing substrates during precisely controlled periods of mitosis is essential for the initiation of anaphase and for exit from mitosis. Given the importance of these reactions, surprisingly little is known about how substrates are recognized by APC/C, perhaps because these molecular interactions are transient and therefore difficult to trace. To overcome this hurdle we have adopted photocrosslinking techniques that allow the detection of weak and temporary interactions, an approach that has been used with great success in studying the secretory pathway and that, in principle, allows the fine mapping of protein-protein interactions down to the resolution of individual amino acid residues (Brunner, 1993; High et al., 1993).

How Are D Boxes Recognized by APC/C?

Our results indicate that the D boxes of cyclin B and of securin are directly recognized by Cdc20 and Cdh1 and are therefore consistent with a number of previous reports that have shown that APC/C substrates can bind to Cdc20 and Cdh1 (Ohtoshi et al., 2000; Burton and Solomon, 2001; Hilioti et al., 2001; Pfleger et al., 2001; Schwab et al., 2001; Sorensen et al., 2001). How specific are these interactions, and what is their biological function?

Three observations argue that the binding of D box substrates to Cdc20 and Cdh1 is specific and important for substrate ubiquitination by APC/C. First, the interactions that we detected were strictly dependent on the D box, a sequence element that is essential for the timely destruction of vertebrate cyclin B and securin (Glotzer et al., 1991; Zou et al., 1999; Hagting et al., 2002). Second, our D box peptides did not only crosslink to purified activator proteins but also to Cdc20 under highly competitive conditions, namely in Xenopus egg extracts whose overall protein concentration is very high (50 μg/ml), but whose Cdc20 concentration is low (2.75–5 μg/ml; [Lorca et al., 1998]), i.e., where Cdc20 only represents about 0.01% of all proteins. Third, and most importantly, mutation of only four evolutionarily conserved amino acid residues in Cdh1 strongly reduced cyclin B binding and at the same time strongly decreased the ability of APC/C to ubiquitinate cyclin B.

Do D Boxes Interact Exclusively with Cdc20 and Cdh1?

In chromatography experiments in which a D box-containing fragment of fission yeast cyclin B was used as an affinity ligand, Yamano et al. (2004) have recently shown that APC/C but not Cdc20 can be isolated from mitotic Xenopus egg extracts. Why do our results differ from the ones obtained by Yamano et al. (2004)? The substrate-activator interactions that we have detected by crosslinking cannot be detected by standard immunoprecipitation experiments (data not shown), indicating that these interactions must be very transient. It is thus not surprising that the affinity chromatography approach used by Yamano et al. (2004) did not reveal Cdc20 binding to D box fragments. It is more difficult to explain why we failed to see D box-dependent binding of substrates to APC/C. It is possible that the peptides used in our experiments interacted weakly but nonspecifically with some component of APC/C, and such interactions may have obscured other interactions that could be D box specific. It is also possible that D box-dependent interactions with APC/C subunits can only be revealed if tandem arrays of D boxes are used, as was the case in the experiments performed by Yamano et al. (2004). In any case, it is important to note that the results by Yamano et al. (2004) and our results are not necessarily mutually exclusive and that there could be multiple interaction sites for D boxes on APC/C and its activator proteins. The latter notion is supported by the finding that D box fragments of yeast cyclins interact with the APC/C subunit Cdc23 in yeast two-hybrid experiments (Meyn et al., 2002) and by the recent observation that Doc1 contributes to recognition of APC/C substrates in a D box-dependent manner (Carroll et al., 2005).

What Is the Function of D Box Binding to Cdc20 and Cdh1?

Ubiquitin chains of a critical length have to be assembled on substrates to allow their efficient recognition by the 26S proteasome (Thower et al., 2000), and ubiquitin ligases are thought to catalyze these chain assembly reactions in a processive manner. Interestingly, Cdh1 mutants whose ability to bind substrates has been reduced are not completely defective in supporting APC/C-mediated substrate ubiquitination but specifically fail to support the conversion of cyclin B into high molecular mass ubiquitin conjugates, implying that Cdh1 is required for processive substrate ubiquitination. Cdh1 therefore shares two important properties with Doc1 (Carroll and Morgan, 2002; Carroll et al., 2005); both proteins promote recognition of D box substrates and are required for their processive conjugation to ubiquitin. However, in contrast to APC/C lacking Doc1, APC/C without Cdh1 (and without any of the other APC/C activator proteins) is completely inactive. We therefore speculate that the ability of APC/C activator proteins to bind substrates is essential for substrate recognition by APC/C.

How Do Cdc20 and Cdh1 Bind D Boxes and Possibly Other Destruction Signals?

We have observed that an amino acid residue that is located in direct proximity to the D box of cyclin B can
be crosslinked to Cdc20 and Cdh1 and that the efficiency of this reaction depends on residues that are predicted to be part of an evolutionarily conserved surface on Cdh1’s propeller structure. Together, these findings indicate that the D box binds directly to one side of the WD40 propeller of APC/C activators (we tentatively refer to this side as the top side). This mode of interaction would be directly analogous to how the WD40 propeller domains of the SCF substrate adaptor proteins Cdc4 and βTrCP bind their substrates (Figure 6; [Orlicky et al., 2003]). This notion is also supported by the observation that three residues that have previously been shown to mediate interactions between Cdh1 and cyclin A-Cdk2 (the RVL motif; [Sorensen et al., 2001]) map to this surface. It is furthermore interesting to note that the evolutionarily conserved area on the top side of the Cdh1 propeller is much larger than the corresponding area on Cdc20 (Figures 3A and S1). Compared to Cdc20, Cdh1 appears to have a broader substrate specificity because it does not only support the ubiquitination of D box-containing substrates but also activates APC/C toward proteins with KEN boxes ([Pfleger and Kirschner, 2000; Burton and Solomon, 2001; Hagting et al., 2002]). It will therefore be interesting to analyze if the top side of Cdh1’s propeller structure contains more evolutionarily conserved residues because it is interacting with multiple destruction signals in APC/C substrates.

Who Interacts First, APC/C with Activator or Activator with Substrate, and Do These Interactions Influence Each Other?

Our crosslinking experiments indicate that Cdh1 directly binds to the TPR domain subunit Cdc27 of APC/C, consistent with our previous findings that Cdh1 cannot bind to APC/C subcomplexes that lack TPR subunits, and that peptides representing Cdh1’s IR tail can bind to recombinant Cdc27 and the related subunit Apc7 in vitro ([Vodermaier et al., 2003]). Our work also provides in vivo evidence that the IR tail is essential for Cdh1 function, which supports the hypothesis that this sequence element is required for APC/C binding ([Passmore et al., 2003; Vodermaier et al., 2003]). It is, however, at first glance surprising that amino acid residues next to the N-terminal C box, next to the C-terminal IR tail, and residues predicted to be part of the WD40 propeller (with the exception of serine 400) can all contact Cdc27. However, it is possible that the C terminus of Cdh1 folds back onto the N terminus, as is the case in Cdc4 and βTrCP, and that multiple contacts exist between Cdh1 and Cdc27. For a number of reasons, we consider it unlikely that the observed binding of Cdh1 to Cdc27 represents an artifact. Most importantly, we found that Cdh1 binding to APC/C is greatly reduced if Cdc27 is deleted, consistent with the previous finding that deletion of the APC9 gene results in the formation of APC/C that lacks both Apc9 and Cdc27 and is defective in Cdh1 binding ([Passmore et al., 2003]). In the future, structural information will clearly be needed to understand exactly how Cdh1 binds to APC/C.

Which APC/C subunits are required for Cdc20 binding is less clear, in part because we have so far not been able to generate Cdc20 probes that can be crosslinked to APC/C. However, the observation that Cdc20 binding to APC/C is also reduced in the absence of Cdc27 (Figure 5D) and the finding that Cdh1 binding to APC/C can be competitively reduced by an excess of Cdc20 (data not shown) raises the possibility that Cdc20 and Cdh1 bind to the same site on APC/C.

In vitro, Cdc20 and Cdh1 can bind to APC/C relatively stably ([Kramer et al., 2000; Kraft et al., 2003]), whereas we have not been able to detect interactions between activators and substrates in the absence of crosslinking agents. These observations argue that substrates that bind to activators in the absence of APC/C may not remain bound long enough to be recruited to APC/C. Instead, it is more plausible to think that first activators will have to associate with APC/C before the binding of a substrate molecule to the activator can lead to substrate ubiquitination.

Our work shows that both types of interactions, between APC/C and activator, and between activator and substrate, can principally occur independently of each other; however, two observations suggest that the interactions may influence each other. The crosslinking of cyclin B peptides to Cdc20 is much more efficient in...
the presence of APC/C than in its absence (Figure 2B), indicating that substrates bind with higher affinity or lower off-rate if Cdc20 is bound to APC/C. For example, as discussed above, it is possible that substrates can interact with multiple surfaces on both APC/C and activators. Furthermore, we have observed that mutation of surface residues in Cdh1 that are required for efficient substrate binding also reduce the ability of Cdh1 to associate with APC/C. It is therefore possible that the binding of activator to APC/C would not only enhance substrate recruitment, but that this vice versa the interaction between substrate and activator also stabilizes the association between activator and APC/C.

Experimental Procedures

Substrate-APC/C Crosslinking

The following peptides were synthesized, dissolved at a concentration of 4 mM in BL (20 mM Tris [pH 7.5], 150 mM NaCl, 0.02% Tween-20), and stored in aliquots in the dark at −20°C (B represents benzophenone-phenylalanine [BPA]; Bachem). Hsa. cyclin B D box peptide, KPGLRPTALDGDIQKVSEQBOAKPMKYC; Hsa. cyclin B D box mutant peptide, KPGLRPTAAADGINKVSEQBOAKPMMP; Kyc. scrambled cyclin B D box peptide, DKQKMLGRVSMKPGPTNEBQAIGRKYC; Hsa. securin D box peptide, ALPKATRKALGT

Experimental procedures, two additional figures, a table, and Supplemental References and can be found with this article online at http://www.molecular.org/cgi/content/full/18/5/543/DC1.

Yeast Methods

All yeast strains used are derivatives of K699 and K700 (ho ade2-1 trpl-1 can1-100 leu2-3,112 his3-11,15 ura3-1 GAL psi kg BPA peptide were incubated in 500 l mitotic egg extract for 5 min at room temperature, followed by 5 min 360 nm light exposure using a 100 W mercury spot lamp (UVP, Upland, California). Either APC/C was immunoprecipitated with Cdc27 antibodies, denatured with 1% SDS, and biotinylated BPA peptides were isolated on monomeric avidin gel (Pierce no. 20228), or biotinylated peptides were isolated from the egg extract directly. Alternatively, 12 μg peptide was incubated with 400 ng of purified recombinant human Cdc20 or Cdh1 (Kramer et al., 2003) for 10 min at 30°C, crosslinked as above, immunoprecipitated with Cdc20/Cdh1 antibodies, and analyzed by Western blotting with “immuno- pure ABC standard” peroxidase staining kit (Pierce no. 32030). For crosslinking to in vitro translated Cdh1, 2.5 μg peptide was incubated for 10 min at 30°C in 5 μl in vitro translation mixture, cross- linked for 5 min, isolated on aigid gel, and analyzed by SDS-PAGE/phosphorimaging.

Cdh1-APC/C Crosslinking

To obtain a higher 35S-methionine labeling of Cdh1, six additional methionine codons were integrated between the cDNA sequences that encode the N-terminal myc tag and Cdh1. Amber mutations were generated by site-directed mutagenesis (Quikchange, Stratagene). In vitro translations were performed using the TNT coupled transcription translation kit (Promega) in the presence of [35S]-Methionine according to the manufacturer’s protocol, except that 1 mM DTT and 100 mM glycine. As a control, DMSO was added in place of the crosslinker. In the case of BPM, chemical crosslinking was followed by UV treatment for 5 min as described above.

Yeast Methods

All yeast strains used are derivatives of K699 and K700 (ho ade2-1 trpl-1 can1-100 leu2-3,112 his3-11,15 ura3-1 GAL psi). A C-ter- minal TAP tag on APC4 and a C-terminal HA tag on APC5 were introduced according to standard procedures. Bypass suppressor strains lacking CDC27 were generated as described (Thornton and Toczyski, 2003), and the loss of CDC27 was confirmed by PCR.

For activator binding assays, TAP-tagged APC was purified by rotating 15 μl IgG sepharose beads with 2 ml yeast lysate for 2–3 hr at 4°C. After washing in IgG wash buffer containing 400 mM NaCl, 12 μl Cdh1 or Cdc20 IVT were added to a total volume of 50 μl IgG wash buffer containing 1 mM DTT and rotated at room temperature for 1 hr. Beads were washed 3× for 5 min with IgG wash buffer containing 2 mM EGTA, and 2× for 5 min with IgG wash buffer containing 1 mM DTT. APC/C was eluted from the beads by addition of 2 μl TEV protease (20 units; Invitrogen) and 15 μl IgG wash buffer containing 1 mM DTT and rotation at room temperature for 2 hr. Eluates, as well as 10% of Cdh1 input and supernatant of the binding reaction were analyzed by SDS-PAGE followed by immunoblotting, and/or phosphorimaging.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, two additional figures, a table, and Supplemental References and can be found with this article online at http://www.molecular.org/cgi/content/full/18/5/543/DC1.

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