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The APC Subunit Doc1 Promotes Recognition of the Substrate Destruction Box

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Summary

Background: Accurate chromosome segregation during mitosis requires the coordinated destruction of the mitotic regulators securin and cyclins. The anaphase-promoting complex (APC) is a multisubunit ubiquitin-protein ligase that catalyzes the polyubiquitination of these and other proteins and thereby promotes their destruction. How the APC recognizes its substrates is not well understood. In mitosis, the APC activator Cdc20 binds to the APC and is thought to recruit substrates by interacting with a conserved target protein motif called the destruction box. A related protein, called Cdh1, performs a similar function during G1. Recent evidence, however, suggests that the core APC subunit Doc1 also contributes to substrate recognition.

Results: To better understand the mechanism by which Doc1 promotes substrate binding to the APC, we generated a series of point mutations in Doc1 and analyzed their effects on the processivity of substrate ubiquitination. Mutations that reduce Doc1 function fall into two classes that define spatially and functionally distinct regions of the protein. One region, which includes the carboxy terminus, anchors Doc1 to the APC but does not influence substrate recognition. The other region, located on the opposite face of Doc1, is required for Doc1 to enhance substrate binding to the APC. Importantly, stimulation of binding by Doc1 also requires that the substrate contain an intact destruction box. Cells carrying *DOC1* mutations that eliminate substrate recognition delay in mitosis with high levels of APC substrates.

Conclusions: Doc1 contributes to recognition of the substrate destruction box by the APC. This function of Doc1 is necessary for efficient substrate proteolysis in vivo.

Introduction

Substrates of the anaphase-promoting complex (APC) [1, 2] generally contain conserved sequence elements called the destruction box (D-box) and KEN-box [3, 4]. Mutation of these elements stabilizes the substrate in vivo and reduces its ubiquitination in vitro, suggesting that these motifs are necessary for recognition by the APC.

Substrate ubiquitination by the APC also requires an activator protein, either Cdc20 or Cdh1 in mitotically dividing cells [1, 2]. Cdc20 and Cdh1 associate with the APC in a cell cycle-dependent manner: Cdc20 binds and activates the APC at the metaphase-to-anaphase transition, whereas Cdh1 maintains activity during late mitosis and G1 [5, 6]. Cdc20 and Cdh1 are also thought to interact directly with target substrates [7–10]. In most but not all cases, the binding of activator to substrate is reduced or eliminated by mutations in the substrate D-box or KEN-box, with Cdc20 often displaying specificity for the D-box and Cdh1 displaying specificity for the KEN-box. This is consistent with a model in which activator proteins bind substrates through conserved motifs and present them to the APC for ubiquitination.

In many cases, however, the behavior of a substrate in vivo cannot be explained simply by its interaction with the activator alone. For example, deletion of the D-box from the budding yeast cyclin Clb2 or the polo-like kinase Cdc5 does not abolish binding of these substrates to Cdh1 (presumably because binding is mediated by the KEN box) but does reduce substrate proteolysis in vivo [10]. Similar observations were made with budding yeast Hsl1 and the human protein Skp1 [7, 11]. One explanation for these results is that D-box recognition is not mediated solely by the activator protein. Consistent with this possibility, a role for the APC itself in substrate recognition has emerged [12–14]. Direct binding has recently been observed between a D-box peptide and APC isolated from mitotic *Xenopus* egg extracts [12]. This interaction is not dependent on activator, suggesting that core APC subunits play a direct and specific role in substrate recognition.

An intriguing candidate for mediating this interaction is Doc1/Apc10, the only core APC subunit thus far implicated in substrate binding [13, 14]. Biochemical analysis of budding-yeast APC shows that Doc1 increases the processivity of substrate ubiquitination by enhancing the affinity of the APC-substrate complex [13]. Importantly, the interaction between APC and the activators Cdh1 and Cdc20 is unaffected by loss of Doc1 function, suggesting that Doc1 promotes substrate binding directly or in concert with other core APC subunits. This possibility is supported by the crystal structures of yeast and human Doc1 [15, 16]. Both structures show that Doc1 contains a single conserved central domain with an overall geometry strikingly similar to that of a number of functionally unrelated proteins. Interestingly, these proteins, including galactose oxidase and sialidase, use the same surface to bind diverse ligands, suggesting that this interface is also used by Doc1 to bind ligands.

We further explored the role of Doc1 in substrate recognition by the APC. Our data demonstrate a requirement for the putative ligand binding interface of Doc1 in D-box-specific substrate binding to the APC. Doc1 mutants that reduce D-box binding to the APC are unable to efficiently target substrates for degradation in vivo, suggesting that the contribution of Doc1 to D-box recognition is critical for APC function in the cell.

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Results

Identification of Residues Required for Doc1 Function

The APC can processively ubiquitinate substrates by transferring multiple ubiquitin molecules to a target protein during a single binding event [13]. The degree of processivity depends on the balance between substrate dissociation and enzyme turnover. The APC subunit Doc1 affects this balance by limiting substrate dissociation [13, 14]. Defects in substrate binding to the APC because of compromised Doc1 function can therefore be detected by a reduction in the processivity of substrate ubiquitination.

To better understand how Doc1 contributes to substrate recognition, we used targeted mutagenesis to identify functionally important regions of Doc1. Sequencing efforts have identified clear Doc1 orthologs in numerous organisms, as well as domains related to Doc1 in other putative ubiquitin ligases that are otherwise unrelated to the APC [15, 16]. We therefore focused our analysis on amino acid positions that are highly conserved in Doc1 orthologs but less well conserved in related "doc-domains" because these residues are the most likely to mediate the APC-specific functions of Doc1. In all, we changed 11 charged or polar residues, either individually or in pairs, to alanine.

Interestingly, the majority of residues selected by these criteria map to the putative ligand binding interface of the protein, consistent with the idea that this region is important for Doc1 function (Figure 1A). However, two of the highly conserved positions, K162 and R163, map to the opposite face, adjacent to where the C terminus of Doc1 exits the core domain. Previous work showed that the C terminus of Doc1 interacts directly with the APC subunit Cdc27 [16], but the functional significance of this interaction has yet to be determined. We therefore also characterized a truncation mutant of Doc1 lacking the C-terminal 28 amino acids.

APC was immunoprecipitated from extracts of yeast cells expressing wild-type or mutant versions of *DOC1* and was tested for its ability to ubiquitinate a radiolabeled N-terminal fragment of sea urchin cyclin B (Figure 1B). The processivity of the reaction was determined by quantifying the molar ratio of ubiquitin to cyclin in the mono-, di-, and tri-ubiquitinated reaction products (Figure 1C). As shown previously [13], APC lacking Doc1 (vector) was almost completely nonprocessive.

Two pairs of point mutations, N238A/H239A and K243A/D244A, displayed significant defects; in particular, the N238A/H239A mutant was able to stimulate processivity only slightly when compared to APC lacking Doc1. In addition, point mutations in K162 and R163 or deletion of the C terminus (CΔ) of Doc1 resulted in modest but reproducible defects in processivity.

Western blotting of the Cdc16 subunit in the immunoprecipitated APC indicated that equivalent amounts of enzyme were included in each reaction (Figure 1B). Importantly, Western blotting of Doc1 showed that the N238A/H239A and K243A/D244A mutants bound APC as well as wild-type Doc1. Levels of the K162A/R163A mutant appeared similar, though slightly reduced, compared to wild-type Doc1. We were unable to determine the amount of the CΔ mutant in these reactions because our antibody specifically recognizes the C-terminal region of Doc1. However, a thorough analysis of the binding of these mutants to APC is described below.

The amino acids required for processive substrate ubiquitination are found in spatially distinct regions of the Doc1 protein; N238, H239, K243, and D244 are all located on a conserved loop on the putative ligand binding interface of Doc1, whereas K162, R163, and the C terminus are located on the opposite face (Figure 1A). We therefore tested the effects of combining mutations that cluster in similar regions of Doc1 (Figures 1B and 1C). Two additional mutants were made: a quadruple point mutant of N238, H239, K243, and D244 to alanine (referred to hereafter as 4A) and a combination of the K162A/R163A mutant and the CΔ mutant. In both cases, these mutant combinations were unable to stimulate processivity above levels displayed by APC lacking Doc1. As observed with the pairs of point mutations, the 4A mutations did not affect the binding of Doc1 to the APC.

Doc1 Mutations that Affect Processivity Fall into Two Classes

The mutations described above define two spatially distinct regions of Doc1. We hypothesized that these regions might affect Doc1 function in different ways. On the basis of our initial characterization and the previously demonstrated interaction between the C terminus of Doc1 and Cdc27 [16], the region of Doc1 defined by mutations in K162, R163, and the C terminus is likely to influence substrate ubiquitination through its interaction with the APC; mutations that inhibit this interaction would result in reduced binding of Doc1 to the APC and lead to an apparent reduction in processivity. In contrast, mutations in the putative ligand binding region of Doc1 appear to specifically reduce the ability of Doc1 to stimulate substrate recognition.

The requirement for these amino acids in Doc1 function was analyzed with purified components in vitro. Wild-type and mutant versions of Doc1 were expressed in bacteria as maltose binding protein (MBP) fusion proteins and purified. Increasing concentrations of recombinant proteins were then added to APC purified from *doc1*Δ cells ($APC^{doc1\Delta}$). Wild-type Doc1 was able to fully restore processivity to $APC^{doc1\Delta}$ with a half-maximal concentration of ~40 nM, whereas MBP alone had no effect (Figure 2A). Doc1 proteins with mutations in residues K162 and R163 or lacking the C terminus were also able to fully restore processivity to $APC^{doc1\Delta}$, but both required increased concentrations to do so. Combining these mutations had an even greater effect: It increased the half-maximal concentration ~30-fold when compared to wild-type Doc1. Nevertheless, at saturating concentrations, even this mutant could stimulate processivity to a level comparable to that of wild-type Doc1 (Figure 2A, bottom right). These data confirm that the ubiquitination defects associated with mutations in K162, R163, and the C terminus are exclusively related to APC binding and not to the ability of Doc1 to stimulate processive substrate ubiquitination. These residues therefore define an APC-interaction surface within Doc1.

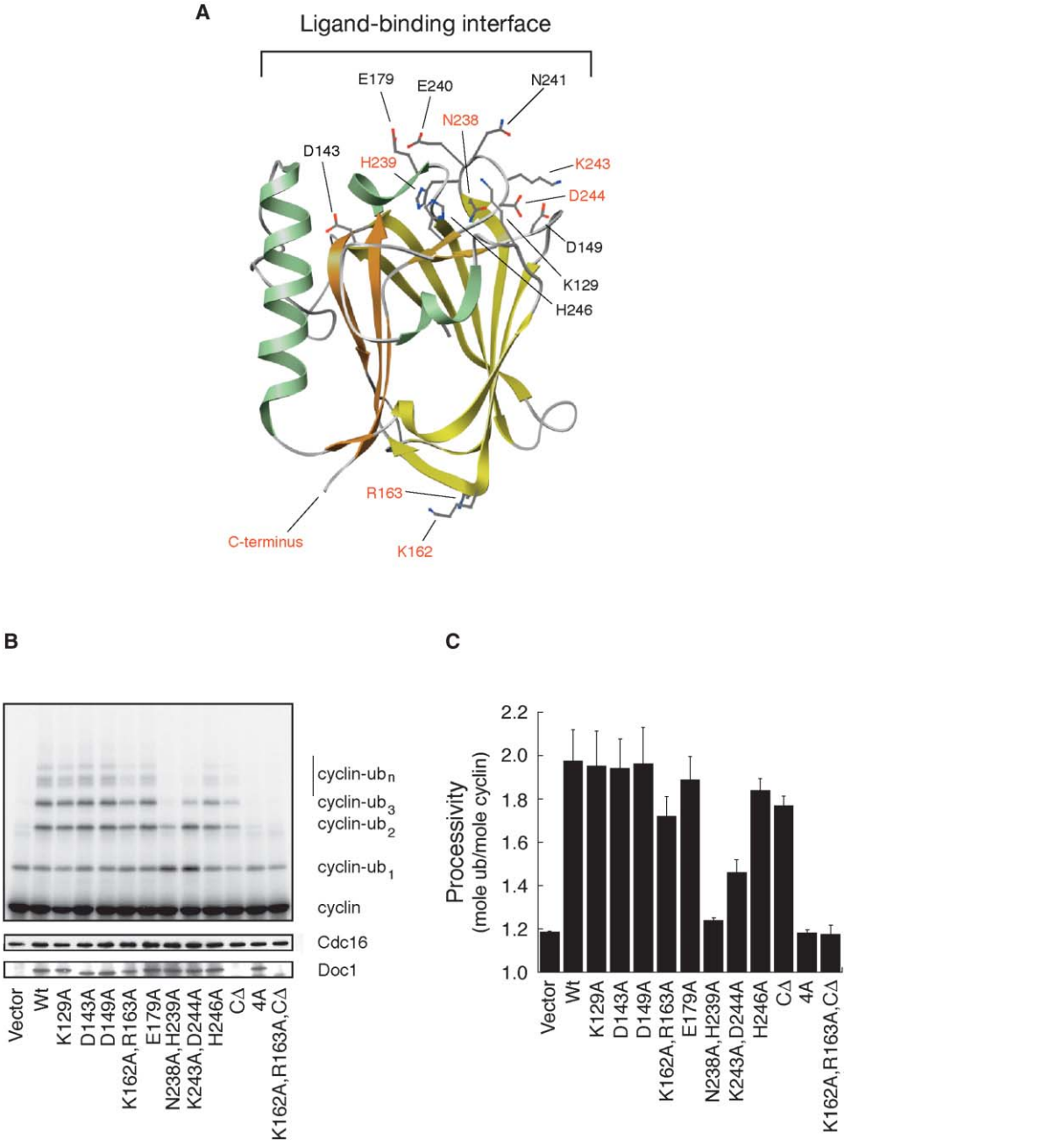

Figure 1. Doc1 Mutants Are Defective in Processivity

(A) The position of highly conserved residues identified by structure-based sequence alignments is shown on budding-yeast Doc1 [15, 16]. Residues that we show are required for Doc1 function are labeled in red. This image was generated with CHIMERA [20].

(B) Doc1 mutants have defects in processivity. APC was immunoprecipitated with anti-Cdc26 antibodies from ~1 mg of extract prepared from strain TC92 (*MAT a, bar1, doc1Δ::URA3*) expressing wild-type *DOC1* (Wt) or various mutants from the *DOC1* promoter on a CEN/ARS plasmid (see Experimental Procedures). Control cells (vector) express no Doc1. Immunoprecipitates were washed three times in lysis buffer and divided in two. One half (top panel) was used for measurement of cyclin ubiquitination activity and visualized with a PhosphorImager. The other half (bottom panels) was used for Western blotting of the APC subunits Cdc16 and Doc1.

(C) Quantification of ubiquitination reactions like those in (B). The numbers of mono (cyclin-ub$_1$)-, di (cyclin-ub$_2$)-, and tri (cyclin-ub$_3$)-ubiquitinated species in each lane were quantified, and the processivity of the reaction was determined by calculating the ratio of ubiquitin to cyclin in the reaction products. Because samples containing fully active Doc1 generate some products above the tri-ubiquitinated species, the data presented are likely an underestimate of the actual ratio in some cases. Error bars represent the standard deviation from three independent experiments.

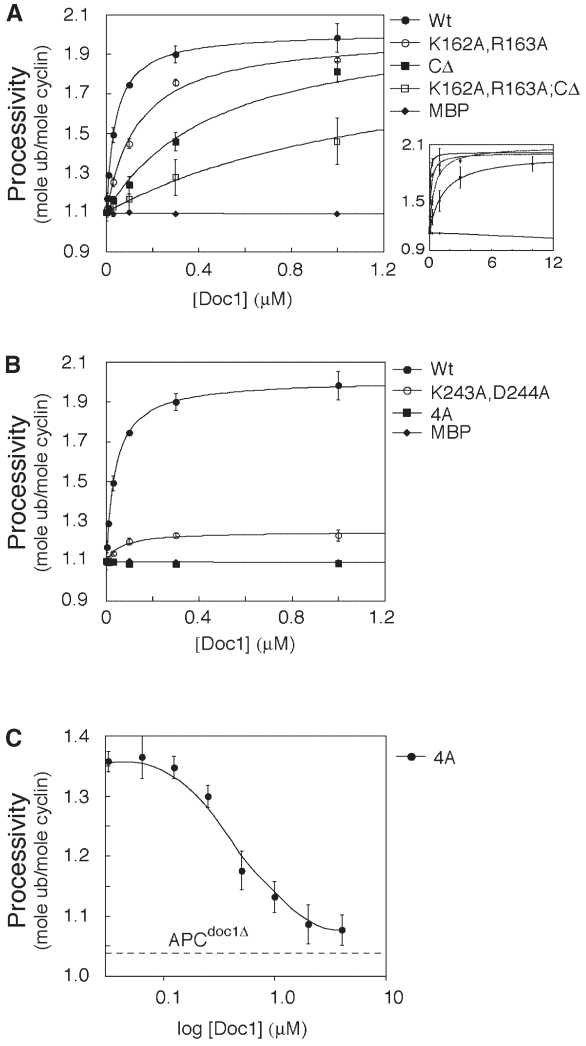

Figure 2. Functional Analysis of Doc1 Mutations that Affect Processivity

(A and B) Doc1 mutants fall into two classes. Increasing concentrations (up to 12 μM) of purified wild-type or Doc1 mutant proteins were added to ~1 nM $APC^{doc1\Delta}$ in complete ubiquitination reactions. MBP alone was used as a negative control. Reactions were allowed to proceed until ~5% of the total substrate was converted to product, and the processivity of the reaction was quantified as described in Figure 1C. Data were fitted to a rectangular hyperbola (solid line). Error bars represent the standard deviation from three independent experiments. ([A], bottom right) The small graph is a scaled version of the large graph and includes additional data at high (>1 μM) Doc1 concentrations.

(C) doc1-4A can bind but not activate the APC. Increasing concentrations of the doc1-4A mutant protein were added to ~1 nM $APC^{doc1\Delta}$ in the presence of ~10 nM wild-type Doc1. Reactions were analyzed as above. Solid line is the best fit of the data. Error bars represent the standard deviation from three independent experiments. The dashed line represents the average processivity of $APC^{doc1\Delta}$ in ubiquitination reactions lacking recombinant Doc1.

In contrast, Doc1 proteins carrying mutations in the putative ligand binding interface were unable to restore full processivity to the reaction. Mutation of residues K243 and D244 to alanine resulted in a Doc1 protein that was only partially able to activate the APC; at saturating concentrations, the ratio of ubiquitin to cyclin in the reaction products was significantly reduced compared to wild-type Doc1 (Figure 2B). Half-maximal stimulation occurred at ~70 nM Doc1, a value comparable to that of wild-type and consistent with the idea that these residues do not influence association with the APC but are specifically required to enhance processivity. The N238A/H239A mutant was a poor activator of $APC^{doc1\Delta}$, making it difficult to accurately determine the concentration required for half-maximal activation. Nevertheless, at no concentration tested (up to 2 μM) was the N238A/H239A mutant able to stimulate processivity above levels seen in Figure 1C (data not shown).

The doc1-4A mutant was unable to activate the APC at any concentration (Figure 2B). To ensure that the mutant protein does bind $APC^{doc1\Delta}$ in these experiments, we asked whether doc1-4A could compete with wild-type Doc1 for APC binding. Indeed, the mutant protein was able to inhibit the ability of wild-type Doc1 to stimulate processivity (Figure 2C). Half-maximal inhibition occurred at ~400 nM doc1-4A, a concentration higher than might be expected if the mutant protein binds APC as well as wild-type Doc1. We suspect, however, that our recombinant doc1-4A protein is not fully active: The yield of mutant protein after purification is lower than that of the wild-type protein, suggesting that the 4A mutations affect the folding or stability of the mutant protein when it is expressed in bacteria. Importantly, at saturating concentrations, the doc1-4A mutant was able to fully inhibit activation of $APC^{doc1\Delta}$ by wild-type Doc1, confirming that the doc1-4A mutant binds but does not activate the APC.

Doc1 thus contains at least two functionally distinct regions. The region associated with the C terminus and residues K162 and R163 is important for the interaction of Doc1 with other APC subunits. In contrast, residues N238, H239, K243 and D244, which map to the putative ligand binding interface of Doc1, appear to be required for the ability of Doc1 to stimulate substrate binding to the APC.

The Putative Ligand Binding Interface of Doc1 Is Required for Substrate Recognition

To further explore the requirement for the putative ligand binding interface in Doc1 function, we purified APC from cells containing wild-type Doc1 or the doc1-4A mutant protein with a tandem affinity purification (TAP)-tagged Cdc16 subunit [13] (Figure 3A). The presence of equal amounts of Doc1 in the two complexes confirmed that the doc1-4A mutation does not affect association with the APC.

As shown previously [13], $APC^{doc1\Delta}$ was defective in binding the sea urchin cyclin B substrate, resulting in the slow production of monoubiquitinated reaction products (Figure 3B). Consistent with our immunoprecipitation data (Figure 1), the activity of purified $APC^{doc1\text{-}4A}$ was indistinguishable from that of $APC^{doc1\Delta}$ despite the presence of normal levels of doc1-4A protein.

To test the generality of the processivity defects caused by doc1-4A, we analyzed a variety of well-characterized yeast substrates, including Pds1 (securin), the mitotic cyclin Clb2, and a fragment of Hsl1 (amino acids

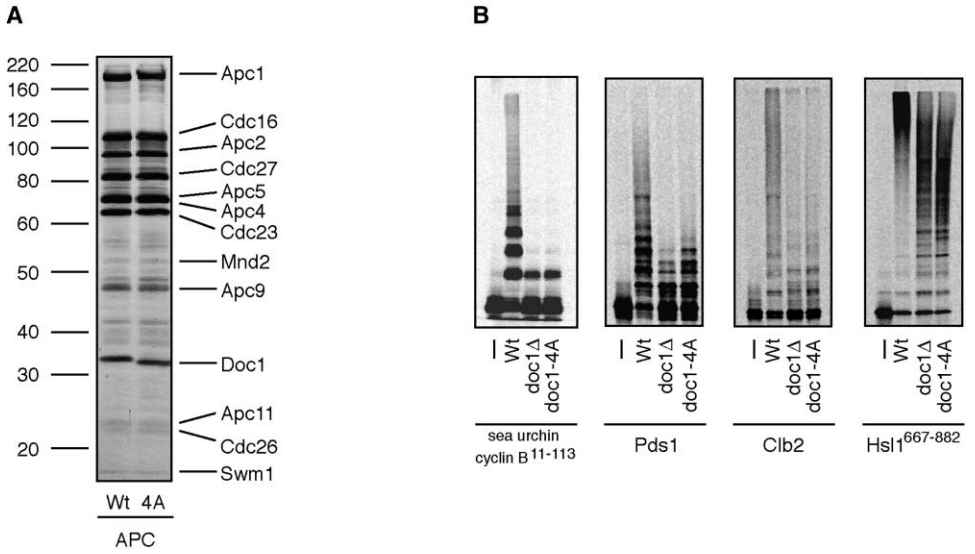

Figure 3. Doc1-4A Mutants Are Defective in Ubiquitination of Multiple Substrates

(A) The ligand binding region of Doc1 is not required for APC assembly. APC was purified in parallel from strains TC130 (*MAT* α, *CDC16::CDC16-TAP:HIS3, doc1Δ::URA3, trp1::TRP1:DOC1*) and TC131 (*MAT* α, *CDC16::CDC16-TAP:HIS3, doc1Δ::URA3, trp1::TRP1:doc1-4A*) with the TAP method [17]. Equal volumes of the final eluate (~20 μl) from the wild-type (Wt) or mutant (4A) APC purifications were separated on a 12.5% polyacrylamide gel, and the proteins were visualized by silver staining. The identity of individual APC subunits is shown on the right; molecular weight is indicated to the left. The doc1-4A mutant protein migrates slightly faster than wild-type Doc1 under these conditions.

(B) $APC^{doc1\text{-}4A}$ is defective for processive substrate ubiquitination in vitro. Complete ubiquitination reactions containing equal amounts (~1 nM final concentration) of wild-type APC (Wt), $APC^{doc1\Delta}$ (doc1Δ), or $APC^{doc1\text{-}4A}$ (doc1-4A) were performed in parallel with the indicated substrates; control reactions (–) have no APC. All substrates were produced in vitro by coupled transcription and translation and are labeled with ^{35}S-methionine. Reactions containing sea urchin cyclin B were resolved on a 15% acrylamide gel. All others were resolved on 7.5% acrylamide gels.

667–882). Interestingly, these substrates, particularly Hsl1, were ubiquitinated much more processively than sea urchin cyclin B, suggesting that yeast APC may bind endogenous substrates with higher affinity than the model cyclin substrate. The ubiquitination of all substrates by $APC^{doc1\Delta}$ was significantly reduced when compared to wild-type APC. Importantly, the activity of $APC^{doc1\text{-}4A}$ was identical to that of $APC^{doc1\Delta}$ toward Hsl1 and Clb2, suggesting that the doc1-4A mutant is completely deficient in the recognition of these substrates (Figure 3B). $APC^{doc1\text{-}4A}$ did have a modest effect on the ubiquitination of Pds1 when compared to $APC^{doc1\Delta}$; however, the activity of $APC^{doc1\text{-}4A}$ was still well below levels exhibited by the wild-type enzyme. Similar results were obtained with all substrates when Cdc20 was used as the APC activator (data not shown). The APC therefore appears to recognize all substrates in a manner that depends on a conserved loop on the ligand binding interface of Doc1.

Doc1 Mediates Substrate Recognition through the Destruction Box

Ubiquitination of Hsl1 by wild-type APC was reduced by introducing point mutations in the substrate D-box or KEN-box (Figure 4A; compare lane 2 with lanes 5 and 8), with the D-box making a greater contribution to ubiquitination than the KEN-box. Importantly, ubiquitination of Hsl1 containing mutations in both the D-box and KEN-box was further reduced (lanes 5, 8, and 11), indicating that the ubiquitination of the single mutant substrates still requires intact APC targeting motifs and, therefore, reflects specific APC activity.

We asked whether the D-box or KEN-box is required for Doc1 to promote substrate recognition. As described above, Hsl1 was less processively ubiquitinated by $APC^{doc1\text{-}4A}$ than by the wild-type enzyme (Figure 4A; compare lanes 2 and 3). Similarly, ubiquitination of the KEN-box mutant substrate by $APC^{doc1\text{-}4A}$ was reduced relative to wild-type APC (lanes 8 and 9). In contrast, ubiquitination of the Hsl1 D-box mutant was unaffected by loss of Doc1 activity, suggesting that Doc1 stimulates processivity only if the substrate contains an intact D-box (lanes 5 and 6).

We also examined the behavior of Clb2 and Pds1 (Figures 4B and 4C). Although Pds1 and Clb2 substrates with mutations in the D-box or KEN-box were ubiquitinated inefficiently, we found that the ability of Doc1 to stimulate ubiquitination was absolutely dependent upon the substrate having an intact D-box; Pds1 and Clb2 that rely only on a KEN-box for recognition were not influenced by Doc1 activity. Doc1 therefore enhances processivity by a mechanism that requires a D-box but not a KEN-box.

Our data also indicate that Doc1 is not solely responsible for recognition of the D-box in these experiments. In all cases, $APC^{doc1\text{-}4A}$-dependent ubiquitination of a wild-type substrate was further reduced by introducing point mutations in the substrate D-box (compare, for example, lane 3 and lane 6 in each panel), suggesting that the D-box promotes substrate ubiquitination through multiple mechanisms, perhaps involving Cdh1.

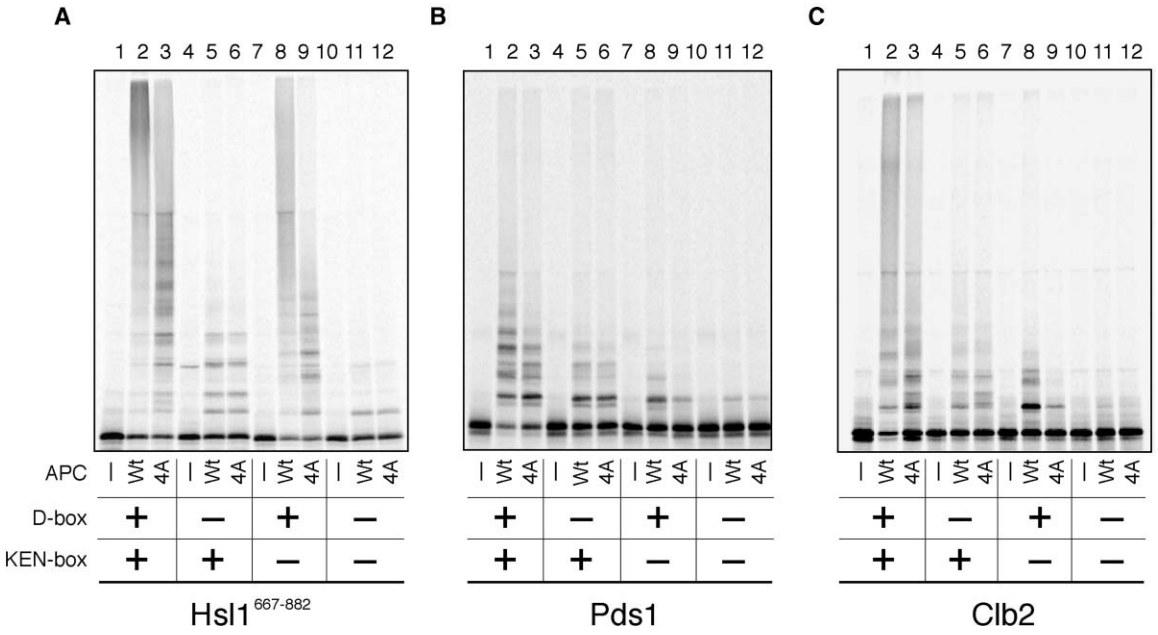

Figure 4. The Substrate D-box Is Required for Recognition by Doc1

Hsl1 (A), Pds1 (B), and Clb2 (C), each containing intact targeting motifs or mutations in the D-box, KEN-box, or both, were included in complete ubiquitination reactions containing no APC (–), wild-type APC (Wt), or APC$^{doc1\text{-}4A}$ (4A). Reactions were performed as in Figure 3, and identical results were obtained in three independent experiments. (+) represents wild-type sequence; (–) represents a mutation. D-box mutations are (RxxLxxxxN → AxxAxxxxA, except Pds1, which is RxxL → AxxA); KEN-box mutations are (KEN → AAA for Clb2 and Pds1, KENxxxE → AAAxxxA for Hsl1).

The Putative Ligand Binding Interface of Doc1 Is Required for Efficient Proteolysis of APC Substrates In Vivo

To determine the specific requirement for the D-box recognition function of Doc1 in controlling mitotic progression, we compared *doc1*Δ cells expressing wild-type Doc1 or the *doc1-4A* mutant through a single, synchronous cell cycle. When released from a G1 arrest, wild-type and *doc1-4A* cells both initiated the cell cycle and entered mitosis with similar kinetics, as suggested by bud emergence and mitotic spindle assembly (Figure 5A; data not shown). However, whereas the wild-type cells rapidly progressed through mitosis and completed cytokinesis about 2 hr after release, the *doc1-4A* mutant population was largely unable to initiate sister chromatid separation or exit mitosis (Figures 5A and 5B). Four hours after release from the α factor arrest, $\sim$70% of the *doc1-4A* cells remained large budded with unseparated nuclei, indicative of a delay at the metaphase-to-anaphase transition.

Western-blotting of the APC substrates Pds1, Clb2, and Ase1 revealed that these proteins were stabilized dramatically in cells that contain the *doc1-4A* mutation (Figure 5C), consistent with a severe defect in APC activity. The inability of the *doc1-4A* mutant APC to efficiently target Pds1 for degradation is likely the cause of the extended metaphase delay. Doc1-dependent recognition of the substrate D-box is therefore critical for APC activity and mitotic progression in vivo.

Discussion

Our results further our understanding of substrate recognition by the APC and support a role for core APC subunits in this process. Specifically, we identified a conserved loop, on the putative ligand binding interface of Doc1, that is required for D-box-dependent substrate binding to the APC and for efficient proteolysis of APC substrates in vivo.

The essential function of the D-box and KEN-box in targeting APC substrates for proteolysis is well established [3, 4]. How these motifs are recognized by the APC is less clear. Abundant evidence from diverse systems suggests that the activators Cdc20 and Cdh1 contribute to substrate recognition and specificity [7–10], and the direct binding of Cdc20 to numerous substrates is D-box dependent. In addition, a direct interaction between purified mitotic *Xenopus* APC and a D-box peptide has recently been demonstrated [12], consistent with the idea that core APC subunits also contribute to substrate recognition.

Our data now demonstrate a function for a specific APC core subunit in D-box recognition. The mechanism by which Doc1 promotes D-box binding remains unclear. Because Doc1 does not affect the affinity of activator binding to the APC [13, 14], it is unlikely that Doc1 influences substrate binding through some effect on the conformation of activators or their binding to the APC. A more likely possibility is that Doc1 acts by enhancing substrate binding to the APC core.

The simplest explanation for our results is that the putative ligand binding interface of Doc1 interacts directly with the substrate D-box. However, using a variety of approaches—from large-scale immunoprecipitation to more sophisticated fluorescence and crosslinking techniques—we failed to detect binding between purified Doc1 and APC substrates. In a heteronuclear single quantum correlation (HSQC) NMR experiment, for exam-

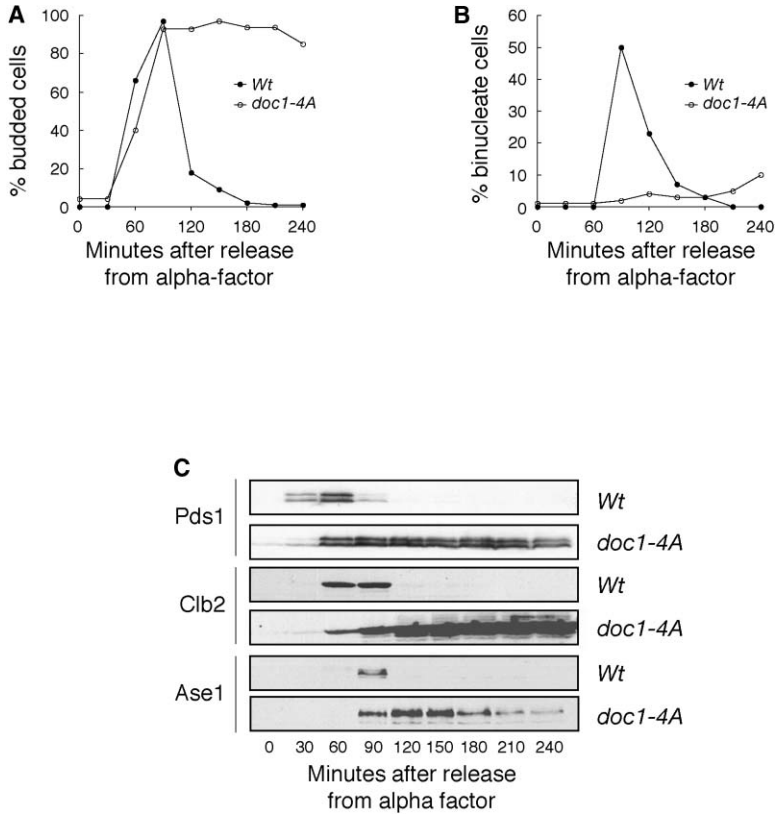

Figure 5. Doc1 Is Required for APC Activity In Vivo

(A and B) The metaphase-to-anaphase transition is delayed in *doc1-4A* cells. Strains TC136 (*MAT a, bar1, doc1Δ::URA3, leu2::LEU2:DOC1, PDS1::PDS1-13myc:HIS3*) and TC137 (*MAT a, bar1, doc1Δ::URA3, leu2::LEU2:doc1-4A, PDS1::PDS1-13myc:HIS3*) were arrested with α factor (1 μg/ml) and released. Samples were taken every half hour and analyzed for cell cycle progression. Budding index (A) and DNA segregation (B) in wild-type (*Wt*) or *doc1-4A* mutant cells were quantified as a function of time after release from G1 arrest. Data are from a representative experiment that was repeated three times. Over 100 cells were counted for each time point.

(C) APC substrates are stabilized in *doc1-4A* cells. 10 μg of cell lysate from each time point was resolved on a 10% acrylamide gel and transferred to nitrocellulose for Western blotting of the indicated APC substrate.

ple, 250 μM Doc1 was incubated in the presence of 500 μM sea urchin cyclin B, and no interaction was detected (X. Luo and H. Yu, personal communication). Thus, if Doc1 does bind the substrate D-box directly, the interaction is of extremely low affinity.

A more likely scenario is that Doc1 alone is not sufficient to mediate target substrate binding. Rather, we suspect that Doc1 requires additional core APC subunits for its activity. For example, Doc1 may form part of a larger binding site that is only present in the context of the intact enzyme. Alternatively, Doc1 may control substrate binding indirectly, perhaps by driving APC structural changes that expose a cryptic D-box binding site. To begin to address these issues, we engineered a version of Doc1 that contained a radio-labeled, photoactivatable crosslinker on a defined position in the ligand binding interface. The protein was then reconstituted into $APC^{doc1\Delta}$, and the crosslinker was activated by photolysis. We were unable, however, to detect the ligand for Doc1, be it a target substrate or additional APC subunits, with this method (data not shown). In addition, experiments similar to those performed by Yamano et. al. [12] did not reveal an interaction between a fragment of Hsl1 immobilized on beads and purified budding-yeast APC (data not shown). Nevertheless, we believe that approaches like these, which examine the interaction of substrates with intact APC rather than individual subunits, will ultimately yield the best understanding of substrate recognition by the APC.

Taken together, the data suggest that the interaction of substrates with the APC is likely to be complex and highly dynamic. One possibility is that substrate association with the activator and APC occurs in a stepwise manner, such that the function of the activator is to load substrates onto the APC for ubiquitination. Alternatively, the interaction of substrates with the APC may involve multiple low-affinity binding sites that display somewhat overlapping specificity.

Experimental Procedures

Strains and Plasmids

All yeast strains used in this study are derivatives of W303. Strains used in each experiment are described in the corresponding figure legends. For site-directed mutagenesis, full-length Doc1 or Doc1 lacking the C terminus (amino acids 1–255) plus 500 bp upstream of the translation start site were cloned into pRS314 (CEN/ARS, *TRP1*). The Quik-change method (Stratagene, La Jolla, CA) was then used to generate the indicated point mutations. All mutants were sequenced to confirm that the desired mutation was the only one present. In some cases, wild-type Doc1 or mutants were subcloned into pRS304 (*TRP1*) or pRS305 (*LEU2*) for integration.

Ubiquitination Assays

Complete ubiquitination assays contained E1, E2, ubiquitin, ATP, Cdh1, and the indicated APC and substrate. All ubiquitination assays were performed as previously described [13]. For immunoprecipitation activity assays in Figure 1, cell lysates were prepared from the indicated strains by bead beating two times for 20 s in lysis buffer (20 mM HEPES [pH 7.4], 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, and 0.1% NP-40). APC was purified by the TAP method, as described [17].

For Figure 1, ^{125}I-labeled sea urchin cyclin was prepared essentially as described [3]. In all other experiments, substrates were produced by coupled in vitro transcription and translation in rabbit reticulocyte extracts, as described by the manufacturer (Promega, Madison, WI). Pds1 and Clb2 are full-length proteins, sea urchin cyclin B is amino acids 13–110, and Hsl1 is amino acids 667–872. All substrates were initially produced with a C-terminal ZZ tag that contains a tobacco etch virus (TEV) cleavage site between the sub-

strate and tag. This facilitates a simple and rapid one-step purification of substrates away from contaminating activities in the reticulocyte lysate. For purification of substrates, one to five reactions were carried out in parallel according to the manufacturer's instructions and pooled. Three volumes of IgG binding buffer (20 mM HEPES [pH 7.4], 150 mM NaCl, and 0.1% NP-40) was added, and this mixture was incubated with 10–50 μl IgG sepharose (Amersham, Uppsala, Sweden) at 4°C for 1 hr. The beads were then washed extensively and cleaved with TEV protease for 10 min at room temperature. Substrates with D-box or KEN-box mutations were amplified by PCR and cloned into the pME34 vector for expression in the reticulocyte extracts. Plasmids bearing mutations in the Hsl1 D-box and KEN-box were a gift from J. Burton and M. Solomon (Yale University, New Haven, CT). Plasmids bearing mutations in the Clb2 and Pds1 D-box and KEN-box were a gift from L. Passmore and D. Barford (Cancer Research UK, London).

Expression and Purification of MBP-Doc1

Wild-type or mutant *DOC1* were cloned into pMal-C2 with PCR products generated from the Doc1 mutant plasmids described above. Plasmid isolates used to express protein were sequenced prior to protein expression to ensure that the desired mutations were the only mutations present. Two liters of BL21 bacterial cells harboring the expression plasmids were grown to mid-log phase at 37°C before the temperature was shifted to 18°C for 1 hr. Protein expression was then induced by addition of 0.3 mM IPTG, and cell pellets were harvested after 16 hr. MBP-Doc1 or the respective mutants were then purified with amylose resin (New England Biolabs, Beverly, MA).

Cell Cycle Analysis

Standard yeast techniques were used [18]. Briefly, strains TC136 and 137 (see Figure 5 legend) were arrested in G1 by addition of α factor (1 μg/ml) for 4 hr at 25°C. α factor was then rapidly washed out and samples were taken every 30 min for 4 hr. α factor was added back after 90% of the cells had budded. DNA was visualized by DAPI staining. Tubulin was visualized by indirect immunofluorescence with antibody YOL1/34. Protein extracts were prepared, and Western blotting was performed as described [19]. Pds1-13myc was detected with a c-Myc (A-14) polyclonal antibody (Santa Cruz Biotech, Santa Cruz, CA) or a 9E10 monoclonal antibody. Clb2 and Ase1 were detected with a polyclonal antibody against the endogenous protein. Ase1 antibodies were a generous gift from D. Toczyski (University of California, San Francisco, CA).

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