Structural analysis of human Cdc20 supports multisite degron recognition by APC/C

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The anaphase-promoting complex/cyclosome (APC/C) promotes anaphase onset and mitotic exit through ubiquitinating securin and cyclin B1. The mitotic APC/C activator, the cell division cycle 20 (Cdc20) protein, directly interacts with APC/C degrons—the destruction (D) and KEN boxes. APC/C^{Cdc20} is the target of the spindle checkpoint. Checkpoint inhibition of APC/C^{Cdc20} requires the binding of a BubR1 KEN box to Cdc20. How APC/C recognizes substrates is not understood. We report the crystal structures of human Cdc20 alone or bound to a BubR1 KEN box. Cdc20 has a disordered N-terminal region and a C-terminal WD40 β propeller with a preformed KEN-box-binding site at its top face. We identify a second conserved surface at the side of the Cdc20 β propeller as a D-box-binding site. The D box of securin, but not its KEN box, is critical for securin ubiquitination by APC/C^{Cdc20}. Although both motifs contribute to securin ubiquitination by APC/C^{Cdh1}, securin mutants lacking either motif are efficiently ubiquitinated. Furthermore, D-box peptides diminish the ubiquitination of KEN-box substrates by APC/C^{Cdh1}, suggesting possible competition between the two motifs. Our results indicate the lack of strong positive cooperativity between the two degrons of securin. We propose that low-cooperativity, multisite target recognition enables APC/C to robustly ubiquitinate diverse substrates and helps to drive cell cycle oscillations.

mitosis | crystallography | multivalency | molecular recognition

The anaphase-promoting complex/cyclosome (APC/C) is a multisubunit ubiquitin ligase that mediates the ubiquitination of a multitude of substrates to drive cell cycle progression (1–3). At the metaphase–anaphase transition, APC/C in complex with its mitotic activator Cdc20 (APC/C^{Cdc20}) ubiquitinates securin and cyclin B1, triggering their destruction to allow separate activation and cohesin cleavage. In anaphase, APC/C bound to the Cdc20 homolog Cdh1 (APC/C^{Cdh1}) further mediate the ubiquitination of cyclin B1 and other mitotic regulators, resulting in their degradation and mitotic exit.

Cdc20 and Cdh1 activate APC/C by contributing to substrate recognition, among other mechanisms (1–3). All APC/C substrates contain one or more of several types of short peptide motifs with highly degenerate sequences, referred to as APC/C degrons. For example, cyclin B1 contains a destruction (D) box with the consensus of RXXLX_{4–5}N (X, any residue), whereas securin contains both a D box and a KEN box (4–6). Biochemical and biophysical studies have established that Cdh1 and the APC/C subunit Apc10 serve as coreceptors for the D box, with the D box bridging an interaction between the two (7–11).

APC/C^{Cdc20} is the molecular target of the spindle checkpoint, which prevents premature sister-chromatid separation and aneuploidy (3, 12–15). In response to kinetochores not properly attached to the spindle microtubules, the spindle checkpoint inhibits APC/C^{Cdc20} using multiple mechanisms. Among these mechanisms, the mitotic checkpoint complex (MCC; consisting of BubR1/Mad3, Bub3, Mad2, and Cdc20) anchors Cdc20 at a site on APC/C that is different from the site bound by free Cdc20, presumably blocking the productive engagement of the D box with Cdc20 and Apc10 (16–21). BubR1/Mad3 contains two KEN boxes (KEN1 and KEN2), both of which are required for the spindle checkpoint (22–25). BubR1/Mad3 has thus been

suggested to inhibit APC/C by acting as a pseudosubstrate, with its KEN boxes competing against those of the substrates for Cdc20 binding (22, 23).

To better understand how APC/C recognizes its substrates, we determined the crystal structures of human Cdc20 alone or bound to the KEN1 box of BubR1, revealing a preexisting KEN-box-binding site at the top of the WD40 β propeller. Structure-based mutagenesis then defined a second conserved surface on the side of the β propeller as a D-box-contacting site. Surprisingly, despite binding to distinct sites on Cdc20, the KEN and D boxes of securin do not apparently interact with APC/C in a highly cooperative manner. The KEN box is largely dispensable for securin ubiquitination by APC/C^{Cdc20}. This lack of strong cooperativity between the two degrons also applies to APC/C^{Cdh1}, but the KEN and D boxes have comparable contributions in this case. D-box peptides inhibit APC/C-mediated ubiquitination of KEN-box substrates, suggesting competition between the two motifs. Our results indicate that the multiple APC/C degrons of securin do not engage APC/C in a highly cooperative manner. A single functional APC/C degron within a substrate supports APC/C^{Cdh1}-dependent ubiquitination. We propose that this low-cooperativity, multisite substrate recognition mechanism expands the substrate spectrum of APC/C, allowing it to attenuate the cellular concentrations of myriad substrates with one or more functional degrons.

Results and Discussion

Structures of Human Cdc20 by Itself or in Complex with BubR1 KEN1.

To determine the structure of human Cdc20, we expressed and purified from Sf9 insect cells a Cdc20 truncation mutant with its N-terminal 60 residues deleted (Cdc20^{ΔN60}). Cdc20^{ΔN60} contained all known functional motifs of Cdc20 (3), including the C box, the KEN box, and the Mad2-interacting motif (MIM), and actively supported APC/C- and D-box-dependent ubiquitination of cyclin B1 (Fig. 1A and Fig. S1). We obtained crystals of Cdc20^{ΔN60} that diffracted to about 3 Å, but could not determine its structure using molecular replacement. We thus made additional Cdc20 truncation mutants Cdc20^{ΔN70} and Cdc20^{ΔN80}, obtained crystals of Cdc20^{ΔN80} that diffracted to 2.0 Å, and determined its structure using molecular replacement with the coordinates of Wdr5 as the search model (Table S1). Only the WD40 domain of Cdc20^{ΔN80} was well defined in the structure. Both the N-terminal region (residues 81–164) and the C-terminal region (residues 477–499; including the IR motif) of Cdc20^{ΔN80} were disordered and not visible in the structure. We then obtained an unrefined structure model of Cdc20^{ΔN60} using molecular

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Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 4GGA, 4GGC, and 4GGD).

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with its KEN box binding to its WD40 domain intramolecularly to limit the access of KEN-box-containing substrates. However, our structure of the free Cdc20 did not support such a model. The N-terminal region of Cdc20 was disordered. The KEN-box-binding site of free Cdc20 did not have unassigned electron density that might have belonged to the Cdc20 KEN box. Deletion of the KEN box of Cdc20 did not alter its ability to activate APC/C (Fig. S1 and see Fig. 4A below).

Comparison of our structures with that of the fission yeast MCC revealed no gross conformational changes of Cdc20 upon binding to Mad2 and the N-terminal TPR domain of Mad3, except for the disordered-to-ordered transition of the Mad2-interacting motif (MIM) of Cdc20 (Fig. 2B) (21). The interactions between human Cdc20 and BubR1 KEN1 were also highly similar to those observed between the fission yeast Cdc20 and Mad3 (Fig. 2B) (21), indicating that the mode of KEN box recognition by Cdc20 was evolutionarily conserved.

Mutagenesis Analysis of the KEN-Box-Binding Site of Cdc20. To validate the functional relevance of the observed interface between Cdc20 and BubR1 KEN1, we mutated the KEN-box-contacting residues of Cdc20 and tested the binding of these Cdc20 mutants to N-terminal fragments of BubR1 (residues 15–237, 20–237, and 15–210) containing the KEN1 box. Binding of BubR1N to Cdc20 was KEN-box-dependent, as a shorter BubR1N fragment (residues 30–210) lacking KEN1 did not bind Cdc20 efficiently (Fig. S2A). Mutations of KEN1-binding Cdc20 residues, including the DYY motif, N329, N331, Q401, and R445, greatly reduced Cdc20 binding to BubR1N (Fig. 2C and D). The T377A mutation had a weaker but still appreciable effect. These results validated the KEN-box-binding site of Cdc20.

Addition of the synthetic BubR1 KEN1 peptide reduced, but did not abolish, Cdc20 binding to BubR1N, indicating that other regions of BubR1N contributed to Cdc20 binding (Fig. S2B). Consistently, mutations of several conserved residues at the top face of the β propeller, including R182 and Y228, also decreased Cdc20 binding to BubR1N (Fig. 2D). These Cdc20 residues might contact the TPR domain of BubR1N, as the corresponding fission yeast Cdc20 residues contacted the Mad3 TPR domain in the yeast MCC structure (21).

Defining the D-Box-Binding Site of Cdc20. Electron microscopy studies have implicated Cdh1 and Apc10 as the coreceptor for the D box (10, 11). It is highly likely that Cdc20 and Apc10 can

also form a coreceptor for the D box. Despite repeated attempts, we could not obtain structures of Cdc20 bound to the D-box peptides of cyclin B1 or securin. In the absence of APC/C, Cdc20 alone might not bind to the D box with appreciable affinity. Indeed, we could not detect Cdc20 binding to beads that were covalently coupled to the cyclin B1 D box peptide. We also could not detect binding between human Apc10 and the D box peptide using either standard pull-down experiments or NMR spectroscopy. Addition of human Apc10 did not promote an interaction between Cdc20 and the D box peptide.

Inspection of the molecular surface of the Cdc20 WD40 domain revealed two conserved patches (Fig. 3A). The first patch at the top of the β propeller is the KEN-box-binding site. A second conserved patch is located between blades 1 and 7 at the side of the β propeller and is formed by residues R174, L176, D177, P179, L202, Y207, W209, and E465 (Fig. 3A and B). Several Cdh1 residues corresponding to these Cdc20 residues were implicated in D-box binding in chemical crosslinking experiments (7). Furthermore, during the many attempts to cocrystallize Cdc20 and D-box peptides, we managed to determine the structure of the Cdc20 WD40 domain to very high resolution (1.35 Å). In this structure, an MPD (2-methyl-2,4-pentanediol) molecule was bound in a conspicuous hydrophobic pocket at the center of this patch (Fig. 3A), suggesting that this pocket might be a functional ligand-binding site. Indeed, in the crystal structure of the fission yeast MCC, a leucine side chain from a neighboring molecule inserted into this pocket in Cdc20 (21). Thus, this second conserved patch is likely involved in D-box binding, with the deep pocket accommodating the leucine in the RXXL consensus motif of the D box.

We next mutated residues in this patch and tested the ability of these Cdc20 mutants to promote cyclin B1 ubiquitination, which was critically dependent on the D box. All these mutations, except for W209A, significantly diminished the ability of Cdc20 to activate APC/C-mediated ubiquitination of cyclin B1 (Fig. 3C). As controls, mutations of the KEN-box-binding residues at the top face of the β propeller, including DYY-AAA, T377A, Q401A, and R445A, had no effects. Furthermore, with the exception of L176A, all ubiquitination-deficient mutants affecting the putative D-box-binding residues still bound to BubR1N (Fig. 2D). In fact, for unknown reasons, D177A, Y207A, and E465A even bound to BubR1N more efficiently than the wild-type Cdc20 did. These mutants were thus correctly folded and presumably conferred weaker APC/C-stimulatory activity due to deficient D-box binding. If so, we would expect that these mutations should not further

Fig. 3. Identification of a conserved D-box-binding site on the side of Cdc20 β propeller. (A) Surface diagram of Cdc20^{WD40}. The conserved Cdc20 residues are colored red. The BubR1 KEN1 peptide (shown as sticks with the KEN motif colored yellow) is grafted onto this structure to indicate the position of the KEN-box-binding site. The MPD molecule bound to a deep pocket is shown as green sticks. (B) Ribbon diagram of Cdc20-BubR1 KEN1 with BubR1 KEN1 and the conserved Cdc20 residues at the D-box-binding site shown as sticks. Residues whose mutations diminish APC/C activity toward cyclin B1 are shown in orange. W209 is shown in gray. (C) The indicated Cdc20 WT and mutant proteins were in vitro translated in rabbit reticulocyte lysate and assayed for their ability to activate the ubiquitination of human Myc-cyclin B1 (1–102) by *Xenopus* APC/C. The reaction mixtures were blotted with anti-Myc. The positions of unmodified cyclin B1 and cyclin B1-ubiquitin conjugates are labeled. (D) Purified Cdc20^{AN60} and Cdc20^{AN60} D177A were assayed for their ability to activate the ubiquitination of human Myc-cyclin B1^{1–102} WT or Δ D by *Xenopus* APC/C. The reaction mixtures were blotted with anti-Myc. The positions of unmodified cyclin B1 and cyclin B1-ubiquitin conjugates are labeled.

a previous report (28), mutation of either the KEN or D box reduced securin ubiquitination, whereas mutation of both largely abolished ubiquitination (Fig. 5A). In the presence of APC/C^{Cdh1}, ³⁵S-securin WT formed higher molecular mass ubiquitin conjugates compared with ³⁵S-securin mK (Fig. 5B, compare lanes 3 and 6). Cold securin WT competed against ³⁵S-securin WT or mK for ubiquitination by APC/C^{Cdh1} more effectively than cold securin mK did (Fig. 5B). These results indicated an involvement of the KEN box in APC/C^{Cdh1}-dependent ubiquitination of securin. The fact that securin mutants with either box mutated still underwent considerable ubiquitination was inconsistent with highly cooperative binding of these two motifs to APC/C^{Cdh1}.

As expected, a synthetic securin KEN-box peptide blocked APC/C^{Cdh1}-mediated ubiquitination of securin mD, a KEN-box substrate (Fig. 5C, compare lanes 3 and 7). Unexpectedly, this KEN peptide also reduced the ubiquitination of securin mK, a D-box substrate (Fig. 5C, compare lanes 2 and 6). Likewise, a synthetic securin D-box peptide not only blocked the ubiquitination of securin mK (compare lanes 2 and 10), but also reduced the ubiquitination of securin mD (compare lanes 3 and 11). These results could be explained by competition between the D and KEN boxes in APC/C^{Cdh1}-catalyzed ubiquitination, although we could not rule out the possibility that the mutated KEN and D boxes still had residual functionality.

To further confirm a competition between APC/C degrons, we tested the effects of several additional synthetic KEN-box and D-box peptides on APC/C^{Cdh1}-dependent ubiquitination of securin mD and two other KEN-box substrates, Bub1⁵²⁰⁻⁶⁵³ and the N-terminal domain of Cdc20. Three KEN-box peptides—Bub1 KEN1, BubR1 KEN1, and securin KEN—inhibited the ubiquitination of these substrates to varying degrees, with Bub1 KEN1 being the most effective (Fig. 5D and E). Consistent with a competition between D and KEN boxes, the cyclin B1 and securin D-box peptides also inhibited the APC/C^{Cdh1}-mediated ubiquitination of all three substrates. A cyclin B1 mutant D-box peptide with the RXXL motif mutated to AXXA did not inhibit Cdc20 ubiquitination (Fig. 5E), demonstrating the specificity of such a competition.

Low-Cooperativity Multisite Substrate Recognition by APC/C. Our structural analysis of human Cdc20, along with the recently published EM structures of APC/C and the crystal structure of the fission yeast MCC (10, 11, 21), firmly establishes that the

APC/C activators, Cdc20 and Cdh1, use two distinct binding sites for binding the two key APC/C degrons: the D and KEN boxes. The KEN-box-binding site lies at the top face of the β propeller, whereas a conserved patch located at the side of β propeller, together with Apc10, forms the D-box-binding site.

Several APC/C substrates, including securin, contain both KEN and D boxes. Highly cooperative bivalent binding of both degrons of a given substrate to APC/C is expected to yield a dissociation constant (K_d) between APC/C and the substrate that is much smaller than either of the two individual K_d s between APC/C and each degron. In this scenario, mutation of either the KEN or D box greatly increases the K_m and impairs securin ubiquitination by APC/C. This does not appear to be the case in APC/C-catalyzed ubiquitination of securin. The KEN box contributes minimally to the ubiquitination of wild-type securin by APC/C^{Cdc20}, but becomes more critical for the ubiquitination of the D-box mutant of securin. Mutation of either box has small effects on securin ubiquitination by APC/C^{Cdh1}. Furthermore, the D-box peptides decrease KEN-box-dependent substrate ubiquitination by APC/C. Taken together, our data are inconsistent with highly cooperative binding of the two degrons of securin to APC/C.

There is little cooperativity between the KEN and D boxes of securin in its ubiquitination by APC/C^{Cdc20}, whereas a weak cooperativity between the two motifs is observed in APC/C^{Cdh1}-dependent ubiquitination. The physical basis for the lack of strong cooperativity is unclear at present. The KEN and D boxes of securin are separated by more than 40 residues. This linker may be too long to optimally span the two degron-binding sites of Cdc20/Cdh1. The gain in binding energy by engaging both motifs may be neutralized by the loss in entropy. Indeed, the linkers between the KEN and D boxes of APC/C inhibitors, Acm1 and Mes1, are substantially shorter and have only around 20 residues (35–37). Both the KEN and D boxes of Acm1 and Mes1 are required for their inhibition of APC/C (35–37). It is possible that the KEN and D boxes in these two proteins have higher cooperativity, allowing them to compete more effectively with bona fide APC/C substrates that have only one degron or have multiple degrons with little cooperativity.

We note that Acm1 is not ubiquitinated by APC/C (35, 36). Even more surprising, deletion of the KEN and D boxes increases its ubiquitination by APC/C, suggesting that the cooperative binding of KEN and D boxes seen in Acm1 might actually hinder ubiquitination (36). Mes1 is both an inhibitor and a substrate of

Fig. 5. Low cooperativity between the KEN and D boxes in APC/C^{Cdh1}-mediated ubiquitination. (A) Ubiquitination of Myc-securin WT, mK, mD, and mDK by APC/C^{Cdh1} with 500 nM UbCh10 for 20 min. The reaction mixtures were blotted with anti-Myc. A contaminating protein in the Myc-securin preparations is indicated by an asterisk. (B) Ubiquitination of ³⁵S-labeled in vitro translated securin WT and mK by APC/C^{Cdh1}. Nonradioactive recombinant purified securin WT and mK were included in the indicated lanes as competitors. The reaction mixtures were resolved on SDS/PAGE and analyzed with a phosphorimager. (C) Ubiquitination of Myc-securin WT, mK, mD, and mDK by APC/C^{Cdh1} with 500 nM UbCh10 for 20 min. The securin KEN- and D-box peptides were added to 1 mM in the indicated lanes. The reaction mixtures were blotted with anti-Myc. (D) Ubiquitination of Myc-securin mD (Left) and Bub1⁵²⁰⁻⁶⁵³ (Right) by APC/C^{Cdh1} for 1 h. The Bub1 KEN1 (1 mM), securin KEN (1 mM), BubR1 KEN1 (307 μ M), and cyclin B1 D-box (153 μ M) peptides were added to the indicated lanes. The reaction mixtures were blotted with anti-Myc. (E) Ubiquitination of in vitro translated Myc-tagged N-terminal fragment of Cdc20 (Cdc20N) by APC/C^{Cdh1} for 2 h. The indicated peptides were added at a final concentration of 150 μ M. The reaction mixtures were blotted with anti-Myc.

APC/C. As a substrate, the KEN and D boxes of Mes1 are not highly cooperative, as mutations of either motif do not impede its degradation *in vivo* and have little effects on its ubiquitination by APC/C *in vitro* (37). So far, there are no known examples of APC/C substrates exhibiting highly cooperative KEN- and D-box-dependent ubiquitination.

Our finding that D-box peptides inhibit the ubiquitination of KEN-box substrates *in trans* suggests a competition between the two motifs in the ubiquitination of certain APC/C substrates. The underlying mechanism of this competition is unclear at present. One possible explanation is that the engagement of the D box with Cdc20/Cdh1 and Apc10 disfavors KEN-box binding to Cdc20/Cdh1, and, to a lesser degree, vice versa. On the other hand, we cannot rule out alternative explanations. For example, the D-box peptides could compete with yet unidentified D-box-like functional elements within the APC/C substrates examined.

Regardless the mechanism, the lack of strong positive cooperativity in degron binding by APC/C means that each degron is capable of supporting substrate ubiquitination and degradation to some degree. This partial functional redundancy generally applies to the cellular degradation of APC/C substrates containing both the D and KEN boxes, including securin, Sgo1, and Mes1 (6, 28, 37, 38). In these cases, mutation of both boxes is required to block substrate degradation. We propose that this functional redundancy ensures the robustness of APC/C-dependent substrate degradation and expands the repertoire of APC/C substrates.

Conclusion

Our structural and biochemical analyses of the mitotic APC/C activator Cdc20 reveal low-cooperativity, multisite binding of APC/C

degrons. This mode of substrate recognition has important implications for the mechanism and regulation of APC/C-dependent ubiquitination reactions. Chemical inhibitors of Cdc20 are expected to cause prolonged mitotic arrest and apoptosis in human cancer cells and thus have therapeutic potential. Our high-resolution structure of human Cdc20 enables virtual screens of compound libraries for such inhibitors.

Materials and Methods

Protein Purification and Crystallization. Human Cdc20 proteins were expressed in insect cells and purified with affinity and conventional chromatography. Standard methodologies were used to crystallize Cdc20 alone or bound to BubR1 KEN1 and determine the structures. See *SI Materials and Methods* for details. Data collection and structure refinement statistics are summarized in Table S1.

Protein Binding and Ubiquitination Assays. Protein binding and ubiquitination assays were performed as described (39, 40). See *SI Materials and Methods* for details.

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