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Analysis of activator-binding sites on the APC/C supports a cooperative substrate-binding mechanism

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Summary

The anaphase-promoting complex or cyclosome (APC/C) is a ubiquitin ligase essential for the completion of mitosis in all eukaryotic cells. Substrates are recruited to the APC/C by activator proteins (Cdc20 or Cdh1), but it is not known where substrates are bound during catalysis. We explored this problem by analyzing mutations in the tetratricopeptide repeat (TPR)-containing APC/C subunits. We identified residues in Cdc23 and Cdc27 that are required for APC/C binding to Cdc20 and Cdh1 and for APC/C function in vivo. Mutation of these sites increased the rate of activator dissociation from the APC/C but did not affect reaction processivity, suggesting that the mutations have little effect on substrate dissociation from the active site. Further studies revealed that activator dissociation from the APC/C is inhibited by substrate, and that substrates are not bound solely to activator during catalysis but interact bivalently with an additional binding site on the APC/C core.

Introduction

Protein ubiquitination is a critical regulatory modification in the control of numerous cellular processes. The final step in ubiquitination is catalyzed by a ubiquitin ligase or E3, which facilitates the transfer of an activated ubiquitin from a ubiquitin-conjugating enzyme (E2) to a lysine on a specific substrate or on ubiquitin itself, thereby forming ubiquitin chains that target the substrate for degradation by the proteasome. The anaphase-promoting complex/cyclosome (APC/C) is a multisubunit E3 required for the initiation of anaphase and the completion of mitosis in all eukaryotic cells (Peters, 2006; Sullivan and Morgan, 2007b; Thornton and Toczyski, 2006). While the most important substrates of the APC/C are securin and the mitotic cyclins (Thornton and Toczyski, 2003), the APC/C ubiquitinates numerous additional substrates involved in late mitotic events. Most of these substrates contain short amino acid sequences, called destruction (D-) boxes and KEN-boxes, that target them to the APC/C.

Ubiquitination by the APC/C is processive: that is, multiple ubiquitins are added in a single substrate-binding event (Carroll and Morgan, 2002). The processivity of ubiquitination is determined by two factors: how long the substrate remains bound to the APC/C active site, and the catalytic rate of the enzyme during that time. Thus, any factor that increases the substrate dissociation rate will cause a decrease in processivity.

APC/C activation requires association with one of two related activator proteins, Cdc20 or Cdh1. Cdc20 activates the APC/C during the metaphase-to-anaphase transition, after which Cdh1 activates the APC/C during mitotic exit and the following G1. Activators are thought to

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recruit substrates to the APC/C. Both Cdh1 and Cdc20 contain a C-terminal WD-40 domain that binds substrates (Burton and Solomon, 2001; Hilioti et al., 2001; Kraft et al., 2005; Schwab et al., 2001). Both activators also contain at least two conserved sequences important for binding to the APC/C core: an 8-residue N-terminal motif called the C-box, and an IR motif at the C-terminus (Passmore et al., 2003; Schwab et al., 2001; Vodermaier et al., 2003).

In addition to recruiting substrates, activator proteins may promote changes in APC/C conformation that increase activity toward those substrates. Evidence for this possibility comes primarily from studies of the unusual APC/C substrate Nek2A, which can bind the APC/C in the absence of activator but is not ubiquitinated unless activator is also added (Hayes et al., 2006). Recent studies reveal that ubiquitination of Nek2A can be stimulated by an N-terminal fragment of Cdc20 containing the C-box motif but lacking the WD-40 domain (Kimata et al., 2008). The C-box region might therefore promote an activating change in APC/C conformation that is independent of the activator's substrate-recruiting function. Consistent with this possibility, electron microscopic (EM) analyses suggest that the APC/C adopts a different conformation in the presence of Cdh1 (Dube et al., 2005).

Two lines of evidence suggest that substrate binding to the APC/C is not mediated entirely by the activator protein. First, there is evidence for a direct, low-affinity interaction between substrate and the APC/C in the absence of activator (Eytan et al., 2006; Yamano et al., 2004). Second, the APC/C subunit Doc1 promotes substrate binding through a mechanism that appears to be independent of activator binding (Passmore et al., 2003). Removal of Doc1 increases the rate of substrate dissociation from the active site, resulting in a decrease in reaction processivity (Carroll et al., 2005; Carroll and Morgan, 2002). Substrate binding to the APC/C might therefore involve contributions from a binding site on the activator and a site on the APC/C core, but the importance of each site during ubiquitination is not known. Since substrate binds the APC/C-activator complex with higher affinity than it binds the APC/C core alone (Eytan et al., 2006; Passmore and Barford, 2005), activator-APC/C binding might trigger a conformational change that enhances substrate-binding affinity, or substrates might be bivalent and interact simultaneously with sites on the activator and APC/C core (Eytan et al., 2006; Passmore and Barford, 2005; Yu, 2007).

The one-megadalton, 13-subunit APC/C is a complex protein machine whose parts are not well understood. A recent analysis of the budding yeast APC/C identified two subcomplexes (Thornton et al., 2006). One subcomplex contains Doc1 and the essential catalytic subunits Apc2 and Apc11, which binds the E2. The other subcomplex contains three large subunits, Cdc16, Cdc23, and Cdc27, each of which contain multiple tetratricopeptide repeats (TPRs). These three subunits associate with the APC/C in series: Cdc23 is the innermost TPR subunit and anchors Cdc16, which anchors Cdc27. A similar arrangement of subunits is supported by EM analysis of APC/C structure (Ohi et al., 2007).

Previous work suggests that the TPR subunit Cdc27 contributes to activator binding to the APC/C: peptides containing the IR motif of activators bind to Cdc27 (Vodermaier et al., 2003), activator can be crosslinked to Cdc27 (Kraft et al., 2005), and removing Cdc27 from the APC/C reduces activator binding affinity (Thornton et al., 2006). However, the APC/C can still be stimulated by activator in the absence of Cdc27, indicating that additional activator-binding sites exist on the APC/C (Thornton et al., 2006).

TPR motifs are thought to mediate protein-protein interactions. A single repeat consists of 34 residues folded into two anti-parallel α-helices, and multiple repeats can pack together to form a protein-binding groove (D'Andrea and Regan, 2003). Each of the TPR subunits of the APC/C has at least nine repeats (an unusually high number (D'Andrea and Regan, 2003)), and stoichiometry calculations suggest that each TPR subunit is present in 2-3 copies on each APC/

C molecule (Dube et al., 2005; Ohi et al., 2007; Passmore et al., 2005). Given that the TPR subunits associate with each other, it appears that the APC/C contains a very large array of TPRs on its surface.

To explore the molecular basis of activator and substrate binding by the APC/C, we constructed and analyzed a series of APC/C mutants with single point mutations in the TPR grooves of yeast Cdc16, Cdc23, or Cdc27. We identified residues in Cdc27 and Cdc23 that are required for activator binding. Detailed biochemical characterization of these TPR mutants, together with studies of the effects of substrate on activator binding, revealed that substrates are not bound solely to activators during catalysis but are shared with a second binding site on the APC/C core.

Results

Identification of residues in the TPR grooves that are important for activator binding

We used a site-directed mutagenesis approach to determine whether the TPR repeats of Cdc16, Cdc23, or Cdc27 mediate the binding of activators or substrates to the APC/C. TPRs have a well-defined structure due to their alpha-helical content, and the residues that lie on the surface of the protein interaction groove can be predicted accurately (Cortajarena et al., 2004; Magliery and Regan, 2005). We focused our analysis on the side chains that were predicted to line the TPR grooves and that were conserved between yeast and humans, as those seemed most likely to mediate binding to other conserved domains.

The three TPR subunits of the budding yeast APC/C contain at least 9 or 10 TPRs each (Figure 1A; sequence alignments provided in Figures S1, S2, S3). The consensus TPR sequence defines the interactions between the two helices A and B in the repeat (Main et al., 2003). Consensus residues create an alternating sequence of large hydrophobic and small residues that interlock to form a helix-turn-helix motif (Figure 1B, C; green residues). Adjacent repeats pack in parallel but at a slightly offset angle, leading to a spiral of anti-parallel helices that form a groove. This groove is lined by residues that are not part of the TPR consensus sequence but instead interact with peptide ligands that bind in the groove. In co-crystal structures of peptides bound to TPR grooves, the residues that most often line the surface of TPR grooves are at positions 2, 6, 9, and 13 of the repeats (Figure 1B, C; pink residues) (Cortajarena et al., 2004; Gatto et al., 2000; Magliery and Regan, 2005; Scheufler et al., 2000). We selected well-conserved residues at these positions in Cdc16, Cdc23, and Cdc27 for alanine scanning by site-directed mutagenesis. We also considered residues at these positions that were conserved between the three yeast proteins (Figure S4). In total, we mutated 10, 21, and 11 residues in Cdc27, Cdc16, and Cdc23, respectively (Figures S1, S2, and S3).

Since mutations that reduce the function of TPR subunits might be lethal, we integrated each mutant gene under the control of its own promoter into a strain of *Saccharomyces cerevisiae* in which the APC/C is not essential and the chromosomal TPR subunit gene is deleted (Thornton and Toczyski, 2003). Using a TAP tag on the Apc1 subunit, we immunopurified each mutant APC/C and divided the preparation for analysis by two different assays: an activity assay to identify enzymatic defects and a Cdh1-binding assay to determine whether any activity defect was accompanied by reduced activator binding. In addition, the APC/C preparation was analyzed by Coomassie Blue staining to asses subunit stoichiometry and the amount of enzyme purified.

In multiple experiments, three mutations in Cdc27 generated reproducible activity defects (Figure 1D): N548A in TPR 5, Y575A in TPR 6, and L579A in TPR 6. N548A (referred to hereafter as Cdc27-A1) and L579A (referred to hereafter as Cdc27-A2) had the most significant defects, and a double mutation of these two residues (Cdc27-A1A2) had greatly reduced

activity, although not as low as that of Cdc27Δ APC/C. Two mutations in Cdc23 generated reproducible activity defects (Figure 1D): N405A in TPR 5 and E442A in TPR 6. N405A (referred to hereafter as Cdc23-A) had a more significant defect and was the only Cdc23 mutation pursued further. Interestingly, Cdc23-A is mutated in the same position in TPR 5 as Cdc27-A1 (Figure S4).

None of the mutations in Cdc27 or Cdc23 disrupted APC/C subunit composition or the levels of the mutant subunits (Figure 1D; bottom panels). Thus, the mutated residues are not required for interaction of TPR subunits with each other or with other subunits of the APC/C.

Importantly, the Cdc27 and Cdc23 mutants with major activity defects all displayed reduced binding to Cdh1 (Figure 1D; middle panels). Thus, the activity defects of the mutant enzymes were accompanied by a reduced affinity for activator.

We tested 20 single mutations in Cdc16. None produced a significant defect in activity, APC/C integrity, or activator binding (Figure S5). In general, the residues facing into the groove at positions 2, 6, 9, and 13 of the repeats are not as well conserved in Cdc16 as they are in Cdc23 and Cdc27, and so it is possible that Cdc16 function depends on other residues in the TPRs.

We also examined the processivity of ubiquitination. As in our previous work (Carroll and Morgan, 2005; Rodrigo-Brenni and Morgan, 2007), we calculated processivity by determining the ratio of the amount of ubiquitin added to the amount of substrate modified. Interestingly, none of the TPR-mutant APC/Cs displayed a significant defect in the processivity of cyclin B ubiquitination (generally about 1.6 ubiquitins/cyclin B). For comparison, Figure 1D (right panel) illustrates the major processivity defect (1.1 ubiquitins/cyclin B) that is observed when substrate affinity is reduced by a mutation in the Doc1 subunit (the Doc1-4A mutant (Carroll et al., 2005)). These results suggest that none of the TPR mutations have major effects on substrate binding during ubiquitination, despite their Cdh1-binding defects.

These experiments, and many of those that follow, were performed with an ^{125}I-labeled sea urchin cyclin B fragment as substrate, because this substrate allows the most accurate measurement of processivity. We obtained similar results in experiments where the substrate was ^{35}S-labeled yeast securin (Pds1) (see Figure 3 below).

A defect in activator binding to the APC/C should result in a shift in the amount of Cdh1 required for half-maximal APC/C stimulation. We therefore purified each mutant APC/C and examined its ability to respond to increasing amounts of Cdh1 (Figure 2). The concentration of Cdh1 required for half-maximal stimulation of wild-type APC/C was about 50 nM, as seen in previous studies (Carroll and Morgan, 2002;Jaspersen et al., 1999). Consistent with their Cdh1-binding defects, the Cdc27-A1, Cdc27-A2, Cdc27-A1A2, and Cdc23-A mutations all caused at least a five-fold increase in half-maximal Cdh1 concentration. Accurate half-maximal values could not be calculated because we could not achieve saturating concentrations of Cdh1 (Figure 2).

We also quantitated the processivity of ubiquitination at each concentration of Cdh1. Wild-type and mutant APC/Cs all had a processivity of about 1.6 ubiquitins/cyclin B (Figure 2), demonstrating again that the defect in activator binding did not result in an apparent defect in substrate residence time at the active site.

Mutations in Cdc27 and Cdc23 affect both Cdc20- and Cdh1-dependent activity

It is not known whether Cdc20 and Cdh1 bind at the same sites on the APC/C. We found that purified Cdc27-A1, Cdc27-A2 and Cdc23-A APC/Cs all displayed significant defects in Cdc20-dependent activity as well as Cdh1-dependent activity in vitro, using ^{35}S-securin as

substrate (Figure 3). These results argue that Cdc20 and Cdh1 interact with the same sites on the TPRs of Cdc27 and Cdc23. We have been unable to develop a Cdc20 binding assay in vitro and so we cannot directly test the affects of these mutations on Cdc20 binding.

Ubiquitination by APC/C^{Cdc20} was generally more processive than ubiquitination by APC/C^{Cdh1} in these assays, and we have observed similar differences in reactions with other substrates (data not shown). Notably, however, longer exposures of these autoradiographs indicate that the Cdc27-A1, Cdc27-A2 and Cdc23-A mutations do not decrease the processivity of securin ubiquitination with Cdc20 or Cdh1 (data not shown).

Mutations in Cdc27 or Cdc23 cause a mild APC/C defect in vivo

To characterize the effects of Cdc27 and Cdc23 mutations in the cell, we used two-step gene replacement to exchange the wild-type genes with mutant alleles at their endogenous loci. None of the mutations had an effect on growth rates in liquid culture at 30°C or 37°C (Figure 4A). Mutant strains were also indistinguishable from wild type when grown on plates (Figure S6).

We also analyzed the *cdc27-A2* and *cdc23-A* strains through a single cell cycle after release from a G1 arrest (Figure 4B). *cdc27-A2* cells displayed only minor defects in mitotic progression, while *cdc23-A* cells exhibited a significant delay: after 195 minutes, 30% of the *cdc23-A* cells had not divided. Both *cdc27-A2* and *cdc23-A* mutants exhibited a decreased ability to degrade the APC/C substrate Clb2, with the defect being more dramatic in *cdc23-A* cells. While delayed mitotic exit in *cdc23-A* cells seems inconsistent with the absence of a growth defect in an asynchronous population, we have seen previously that yeast with mild APC/C mutations show more significant cell-cycle defects following an alpha-factor release (Carroll et al., 2005). These cells are clearly able to degrade enough securin to separate their sister chromatids, and we presume that excess Clb-Cdk1 activity is inhibited by the Cdk1 inhibitor Sic1. These phenotypes are therefore characteristic of cells with moderate defects in APC/C activity.

We also found that the *cdc27-A2* and *cdc23-A* strains are resistant to the growth defects that result from overexpression of *CDH1* or *CDC20* (Figure S6). Thus, mutations in the TPR grooves of Cdc27 or Cdc23 reduce the ability of the APC/C to bind Cdc20 and Cdh1 both in vitro and in vivo, further demonstrating that both activators bind to these sites on the APC/C.

cdc27-A2 cdc23-A double mutant cells arrest in metaphase with high levels of mitotic cyclins

To analyze the combined effects of mutations in Cdc27 and Cdc23, we mated a *cdc27-A2 MAT*α strain to a *cdc23-A MAT*a strain. Tetrad dissection of spores from the resulting diploid strain indicated that the *cdc27-A2* and *cdc23-A* mutations are synthetically lethal. As Cdh1 is not essential for viability, this lethality is likely to be due to defective Cdc20 binding. This result is consistent with our data showing that the mutations in Cdc23 and Cdc27 affect Cdc20-dependent APC/C activity in vitro (Figure 3).

To determine the terminal phenotype of the double *cdc27-A2 cdc23-A* mutant, we used the conditional expression of a wild-type copy of *CDC23* to allow integration of both the *cdc27-A2* and *cdc23-A* mutations into the same strain. We integrated wild-type *CDC23*, *cdc23-A*, or an empty vector at the *ADE2* locus of a strain where the chromosomal copy of *CDC23* had been placed under the control of the P_{GAL1} promoter and *cdc27-A2* had replaced *CDC27* at the endogenous locus. These strains were arrested in alpha factor in the presence of galactose. Glucose was added to suppress transcription of the chromosomal *CDC23* for two hours before releasing from the arrest into media containing glucose.

Cells carrying an integrated copy of *CDC23* displayed minimal defects like those seen in the *cdc27-A2* strain (Figure 4B, C). However, cells in which the *cdc23-A* gene was integrated

(resulting in the *cdc27-A2 cdc23-A* double mutant) arrested with large buds, single DNA masses, and short metaphase spindles. The same phenotype was observed in cells carrying an empty vector (resulting in a *cdc27-A2 cdc23Δ* double mutant). Both the *cdc27-A2 cdc23-A* and *cdc27-A2 cdc23*Δ strains accumulated large amounts of the APC/C substrate Clb2. Thus, the *cdc27-A2 cdc23-A* double mutant phenotype is the same as an APC/C-null phenotype, demonstrating that without at least one of the two TPR subunits to bind activator, the APC/C cannot ubiquitinate substrates efficiently enough to allow progression into anaphase.

The IR motif of Cdh1 interacts with the TPR groove of Cdc27, but the C-box binds elsewhere

To determine whether the well-characterized APC/C-interaction motifs on Cdh1 interact with the TPR grooves of Cdc27 or Cdc23, we tested the ability of Cdh1 lacking either its C-box or IR motifs to activate wild-type and TPR-mutant APC/C. Mutation of either the C-box or IR motifs in Cdh1 causes a defect in APC/C activity. If an activator motif interacts with the TPR residues that are changed in our mutants, then we would expect to see no further activity defect when activator and TPR mutations are paired together.

We mutated the C-terminal IR residues of Cdh1 to AA (ΔIR), and used a previously characterized C-box mutant (I58A P59A, referred to here as the ΔC-box mutant) (Thornton et al., 2006). The ΔIR and ΔC-box mutations each caused a large reduction in wild-type or Cdc27-A2 APC/C activity (Figure 5A; normalized in Figure 5B for the amount of enzyme added and background activity). Importantly, Cdh1-ΔIR stimulated wild-type and Cdc27-A2 APC/Cs to a similar extent. Thus, in the absence of the IR motif on Cdh1, the Cdc27-A2 mutation had no further defect, implying an interaction between the two. However, since mutation of the IR motif on Cdh1 still reduced activity of Cdc27-A2 APC/C, there must be other residues on the APC/C that are important for IR binding.

In contrast, the Cdh1-ΔC-box stimulated wild-type APC/C but did not generate detectable activity with Cdc27-A2 APC/C. The C-box is therefore essential in the Cdc27-A2 mutant, suggesting that the C-box binds elsewhere to help generate the remaining APC/C activity in this mutant.

In the Cdc27-A1A2 mutant, all IR-dependent activity was eliminated (Figure 5C, D). It is therefore unlikely that there are other significant IR-binding sites on the APC/C. These results are consistent with previously published experiments demonstrating that the IR interacts with Cdc27 but the C-box does not (Thornton et al., 2006). Additionally, the lack of a growth defect in the Cdc27-A1A2 strain despite the loss of all IR-dependent activity (Figure 4A) is consistent with previous evidence that mutation of the IR motif in Cdc20 has little apparent effect in vivo (Thornton et al., 2006).

Mutation of either the IR motif or C-box greatly reduced the activity of the Cdc23-A mutant (Figure 5E, F), suggesting that these motifs do not interact with the residue mutated in Cdc23-A. We presume that the ΔIR mutations reduced activity in this mutant because the interaction with Cdc27 was lost (Figure 5A and 5B), and the ΔC-box mutation reduced activity because the C-box interacts with another, unidentified site. Therefore, the residue mutated in Cdc23-A is likely to interact with a third, unknown site on Cdh1. When the Cdc23-A mutant is combined with either the ΔC-box or the ΔIR mutation, two out of three interactions are disrupted, reducing the affinity to a level that is too low to allow detectable ubiquitination in our assays.

Detailed analysis of ΔC-box and ΔIR mutants revealed that these mutations have no major effects on the processivity of substrate ubiquitination (Figure S7). These results are consistent with our evidence that processivity is not affected when activator-APC/C affinity is reduced by mutations in Cdc27 or Cdc23.

TPR mutations increase the rate of activator dissociation from the APC/C

To obtain a more detailed understanding of the activator-binding defects of TPR-mutant APC/Cs, we measured the rate at which radiolabeled Cdh1 dissociates from immunopurified APC/C (Figure 6A). Cdh1 dissociated from wild-type APC/C with a half-time of 25 min (k_{off} = 4.6×10^{-4} s^{-1}) and dissociated more rapidly from the TPR mutants: the half-time was 10 min (k_{off} = 1.1×10^{-3} s^{-1}) with Cdc27-A2 APC/C and 3 min (k_{off} = 3.8×10^{-3} s^{-1}) with Cdc23-A APC/C. These results support two conclusions: first, that activator binds the APC/C with very high affinity; and second, that decreased activator affinity in the TPR mutants is due, at least in part, to an increased dissociation rate.

We also measured the rate at which substrate (^{125}I-cyclin B) dissociates from immunopurified APC/C-Cdh1 complexes. Substrate dissociated from the APC/C with a half-time of ~1 min (an average from three experiments, apparent k_{off} = 0.01 s^{-1}) (Figure 6B). This is more rapid dissociation than that of Cdh1 from wild-type APC/C, suggesting that activator remains bound to the APC/C for a longer time than substrate. Multiple substrates might therefore be modified during a single activator-binding event. This is not the case with Cdc23-A APC/C, from which substrate and activator dissociate at roughly similar rates.

We also determined the dissociation rate of Cdh1 from wild-type and Cdc23-A APC/C in the presence of E1, E2, ATP and ubiquitin, and found that the presence of these reaction components had no effect on dissociation rate (Figure S8A). Similarly, these components had no significant effect on the rate of substrate dissociation (Figure S8B).

Substrate promotes activator-APC/C binding through a bivalent interaction

We were surprised to observe that APC/C mutants with an increased activator dissociation rate do not display a processivity defect (Figure 2). If substrate is bound solely to the activator during ubiquitination by these mutants, then that substrate should also have an increased dissociation rate and might therefore be ubiquitinated less processively. The simplest explanation for our results is that substrate is not bound only to activator during the reaction but is passed to and/or shared with a second, activator-independent site on the APC/C. Given that many APC/C substrates contain multiple degradation motifs, an appealing possibility is that a single substrate binds in a bivalent fashion to sites on the activator and the APC/C core. If this were the case, a reduction in activator-APC/C affinity in our TPR mutants would not necessarily have a significant impact on substrate residence time in the active site.

If a substrate binds simultaneously to both activator and APC/C, then it should increase activator-APC/C binding affinity. This possibility has not been tested previously in biochemical detail, but it has been shown that overexpression of the APC/C substrate Hsl1 increases the amount of Cdh1 bound to the APC/C in vivo (Burton et al., 2005). On the other hand, cyclin substrate had no apparent effect on activator-APC/C binding in native-gel binding assays (Passmore and Barford, 2005), and our own results (Figure 6A) show clearly that activator binds very tightly to the APC/C in the absence of substrate. It remained possible, however, that substrate does enhance the already high activator-APC/C affinity but that measurement of this effect requires sensitive analyses of rates of activator dissociation from the APC/C.

We therefore analyzed the effects of substrate on the half-time of Cdh1 dissociation from the APC/C. The addition of 50 μM cyclin prolonged the apparent half-life of activator bound to wild-type APC/C by about 10-fold (Figure 6C). Similarly, substrate caused a significant extension of the half-life of activator bound to the Cdc23-A APC/C, from 3 min (apparent k_{off} = $3.8\text{x}10^{-3}$ s^{-1}) in the absence of substrate to 12 min (apparent k_{off} = $9.5\text{x}10^{-4}$ s^{-1}) in the presence of 50 μM cyclin. Thus, under these conditions, substrate significantly reduces the

apparent rate of activator dissociation, as expected for the bivalent substrate-binding mechanism.

Using our standard activator binding assay rather than half-life measurements, we tested the effects of varying concentrations of the APC/C substrates securin, cyclin, and Hsl1 (Burton and Solomon, 2001) on Cdh1-APC/C binding. The high binding affinity of Cdh1 for wild-type enzyme made the stimulation of activator binding difficult to observe in this assay, but all three substrates clearly promoted Cdh1 binding to Cdc23-A APC/C (Figure 7A). Cyclin and securin stimulated activator binding to the Cdc23-A APC/C at micromolar concentrations, and the effect of cyclin was abolished by mutation of its D-box sequence. Hsl1 was more effective, stimulating activator binding at far lower concentrations (tens of nanomolar). Mutation of APC/C-recognition sequences in Hsl1 prevented its effects. The potency of Hsl1 in this assay is consistent with previous evidence that Hsl1 is the most processively-modified APC/C substrate known and might therefore possess a particularly high affinity for the APC/C active site (Carroll et al., 2005).

If a bivalent substrate interaction promotes activator binding, then the stimulation of activator binding might be reduced at high substrate concentrations, where the two substrate-binding sites could be occupied by two separate substrate molecules rather than a single bridging molecule. Indeed, the high-affinity substrate Hsl1 displayed this behavior: its stimulatory effects on activator binding were not observed at the highest concentrations tested (Figure 7A).

The bivalent substrate-binding model requires a substrate-binding site on the core APC/C. There is substantial previous evidence that a core binding site exists and depends on the subunit Doc1. Mutation of Doc1 is known to reduce substrate binding and reaction processivity but does not appear to affect activator binding or the concentration of activator required for half-maximal stimulation of APC/C activity (Carroll and Morgan, 2002; Passmore et al., 2003). Using our substrate-dissociation assay, we confirmed that substrate binds very poorly to Doc1-4A APC/C (Figure S9). Thus, if the stimulation of activator-APC/C binding by substrate depends on a bivalent bridge between the activator and the Doc1-dependent core binding site, then it should be abolished by mutation of Doc1.

We therefore analyzed the dissociation rate of activator from Doc1-4A APC/C. Mutation of Doc1 had no effect on activator half-life (Figure 7B), consistent with previous indications that Doc1 does not promote substrate binding through an effect on the activator. Mutation of Doc1 had a striking effect in the presence of substrate: high concentrations of cyclin did not prolong activator binding in the absence of Doc1 function (Figure 7B). These results support the model that substrate promotes activator binding by providing a bivalent bridge between a site on the activator and a Doc1-dependent site on the APC/C core.

Discussion

Our results provide new insights into the mechanism of substrate binding by the APC/C. We identified mutations in the APC/C that increase the rate of activator dissociation but do not significantly affect reaction processivity, suggesting that substrates are not bound solely to the activator during ubiquitination. Further analysis of activator binding by mutant and wild-type APC/Cs then revealed that the affinity of activator for the APC/C is enhanced in the presence of substrate, probably as a result of a bivalent bridging interaction between a substrate-binding site on the activator and another on the APC/C core.

Our results, as well as most previous studies of APC/C substrate-binding mechanisms, are most readily explained by a model diagrammed in Figure 7C and explored in more detail in Figure S10. According to this scheme, activator, substrate, and the APC/C core form a trimolecular complex in which each of the three components interacts with both of the others. According

to established principles of cooperative binding in trimolecular complexes (Goodrich and Kugel, 2007), this model can explain why defects in activator-APC/C affinity have only minor effects on reaction processivity. The model predicts that substrate residence time on the APC/C (and thus reaction processivity) is determined by the rates of dissociation of (1) substrate from the activator-APC/C complex and (2) activator-substrate complex from the APC/C. Based on reasonable estimates of binding affinities (Figure S10), we predict that the first of these dissociation rates is about 200-fold greater than the second. Thus, the first rate is the primary determinant of the overall substrate dissociation rate. In our TPR mutants, where activator-APC/C affinity is reduced, there should be no change in the first rate and perhaps a ten-fold increase in the second, so that the unchanged first rate is still the primary determinant of substrate dissociation rate. In contrast, inactivation of the APC/C-substrate binding site by mutation of Doc1 is predicted to increase both substrate dissociation rates, explaining the major processivity defect of Doc1 mutants. This model can also explain why processivity of securin ubiquitination with Cdc20 is higher than that with Cdh1 (Figure 3): we speculate that Cdc20 has a higher affinity for certain substrates, which would reduce the first substrate dissociation rate and could thus significantly prolong substrate residence time in the active site.

Our model is also consistent with the fact that many, if not all, APC/C targets contain multiple APC/C-recognition motifs. Although the well-established D- and KEN-box sequences are clearly required in many cases for APC/C-dependent ubiquitination, they are often not sufficient, suggesting that many substrates contain additional, as yet unidentified, APC/C-recognition sites. Numerous APC/C substrates contain unconventional targeting motifs (see Sullivan and Morgan, 2007a, and references therein), and so there is a clear potential for substrates to employ multivalent interactions with sites on both the activator and APC/C.

Recent studies suggest that the N-terminal C-box region of the activator induces a conformational change in the APC/C that enhances the rate of substrate ubiquitination (Dube et al., 2005; Kimata et al., 2008). This possibility is not incompatible with our model: the activator might help link the substrate to the APC/C core while also triggering a conformational change that somehow promotes ubiquitin transfer from the E2 to the substrate.

Our studies, as well as previous work (Passmore and Barford, 2005; Thornton et al., 2006), indicate that activator binds the APC/C with high affinity in the absence of substrate; substrate boosts this affinity but is not required for high-affinity activator binding. These results are not consistent with previous speculation that substrate binding to activator is a prerequisite for activator binding to the APC/C (Burton et al., 2005). Instead, our results argue that activator can associate with the APC/C in the absence of substrate and might even remain bound during multiple rounds of substrate binding and dissociation. On the other hand, we also appreciate that the activator (and substrate) dissociation rates measured in our experiments might not be accurate reflections of these rates inside the cell, where they could be regulated by additional factors.

Mutation of conserved residues predicted to line the TPR grooves of the APC/C revealed that TPR motifs mediate the binding of both Cdc20 and Cdh1 to the APC/C. Our evidence for an IR-binding site on Cdc27 is consistent with previous work showing that the IR motif of Cdh1 interacts with Cdc27 (Kraft et al., 2005; Thornton et al., 2006; Vodermaier et al., 2003). Activator binding to Cdc23, however, has not been reported. Our results do not indicate what part of the activator binds to the TPR motif of Cdc23. TPR grooves tend to bind extended peptides, and so Cdc23 could bind a disordered loop or the N-terminus of the activator. Our results also do not identify the C-box binding site, but previous results suggest that a C-box binding site might exist on Apc2 (Thornton et al., 2006). Additional support for this idea comes from EM structures that locate Apc2 in close proximity to bound Cdh1 (Dube et al., 2005; Ohi et al., 2007).

The role of the TPRs in Cdc16 remains unclear. The removal of Cdc16 from Cdc27Δ APC/C abolishes the remainder of its activity, even though Cdc23 remains in place (Thornton et al., 2006). Cdc16 might simply stabilize Cdc23 or other essential subunits, or activators may bind to Cdc16 at sites that we did not mutate. While previous work showed some binding of IR peptides to Cdc16 (Vodermaier et al., 2003), our work shows that the Cdc27-A1A2 mutations are sufficient to eliminate IR binding.

Of the many recently proposed models of APC/C-substrate recognition (reviewed by Yu, 2007), we support 'multivalency' models in which substrate is shared by binding sites on the activator and APC/C core, perhaps accompanied by an activator-induced conformational change that promotes activity. In these models, the activator provides additional substrate-binding sites that enhance weak interactions between substrate and the APC/C core. These multivalent mechanisms could also enhance the fidelity of substrate recognition by requiring multiple substrate-binding steps, or provide the APC/C with greater flexibility in substrate recognition by allowing the combinatorial integration of multiple degradation motifs that interact separately with the activator and the APC/C. Thus, these mechanisms might allow the APC/C to recognize more substrates with greater specificity, and might provide an important level of proofreading for an enzyme whose substrates are irreversibly destroyed.

Experimental Procedures

Strains and Plasmids

All yeast strains are in a W303 background and are listed in Table S1. Details of strain construction are provided in Supplemental Experimental Procedures.

Ubiquitination and Cdh1 binding assays

APC/C was purified using a C-terminal TAP tag on Apc1 as described (Thornton et al., 2006), except for Doc1-4A APC/C, which has a C-terminal TAP tag on Cdc16 (Carroll et al., 2005). Ubiquitination assays contained Uba1, Ubc4, ubiquitin, ATP, APC/C, activator, and substrate, prepared as described (Carroll and Morgan, 2005; Rodrigo-Brenni and Morgan, 2007) and detailed in Supplemental Experimental Procedures. All in vitro translation was performed with the TNT Quick-Coupled Transcription/Translation System (Promega, Madison WI) in rabbit reticulocyte lysate.

For analysis of APC/C activity in immunoprecipitates as in Figure 1, cell lysates were prepared from a ~300 mg cell pellet of the indicated strains by bead-beating (see Supplemental Experimental Procedures). Lysates were incubated with IgG-coupled Dynabeads for 2 h at 4° C, and beads were washed in lysis buffer before 10% of the preparation was removed, washed with QAH buffer (20 mM Hepes pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$), and resuspended with E1, E2, ATP, ubiquitin, insect cell-derived Cdh1 and ^{125}I-cyclin B for 30 minutes. A limiting amount of Cdh1 was used to normalize for the amounts of immunopurified APC/C.

For analysis of Cdh1 binding, 90% of the immunopurified APC/C was incubated with 10 μl in vitro translated ^{35}S-Cdh1 and 50 μl HBST (10 mM Hepes pH 7.4, 150 mM NaCl, 0.1% Triton X-100) for 1 h at room temperature. Immunoprecipitates were washed three times before separation by SDS-PAGE. The gel was stained with Coomassie Blue before visualization with a PhosphorImager.

For Cdh1 dose responses (Figure 2), wild-type and mutant APC/C were purified in parallel using the full TAP purification protocol (Carroll and Morgan, 2005). Insect cell-derived Cdh1 was added in increasing concentrations.

For Cdc20-dependent APC/C assays (Figure 3), Cdc20 was purified from rabbit reticulocyte lysate (see Supplemental Experimental Procedures) and added to APC/C reactions containing purified ^{35}S-securin and wild-type or mutant APC/C that had been purified in parallel by the full TAP purification procedure (Carroll and Morgan, 2005).

For ΔC-box and ΔIR mutant Cdh1 assays (Figures 5 and S7), wild-type or mutant activator was translated in vitro and added directly to ubiquitination assays in the translation mix, as these mutants have such weak activity that they often do not survive purification. Equivalent amounts of reticulocyte lysate without in vitro translated Cdh1 were added to control reactions.

For analysis of the stimulation of Cdh1 binding to APC/C by substrate (Figure 7A), Cdc23-A APC/C was immunopurified using IgG-coupled Dynabeads and incubated with 10 μl in vitro translated ^{35}S-Cdh1 and 50 μl HBST containing varying concentrations of APC/C substrates for 1 h at room temperature. Immunoprecipitates were washed two times for 30 s with HBST and samples were separated by SDS-PAGE. Gels were stained with Coomassie Blue to confirm that equivalent amounts of APC/C were immunopurified in each lane. The cyclin substrate used in these experiments is the sea urchin cyclin fragment described above and its D-box mutant (N50A). The securin substrate is a fragment of yeast Pds1 (residues 1-110), containing both a KEN- and D-box, that was expressed in bacteria. The Hsl1 substrate is a bacterially-expressed MBP fusion of an Hsl1 fragment (residues 667-872) (Burton and Solomon, 2001). We also tested the D/KEN-box double mutant (RAALSDITN starting at residue 828 changed to AAAASDITA, and KEN starting at residue 775 changed to AAA) (Burton and Solomon, 2001).

Cell cycle analysis

For Figure 4B, strains were grown at 30°C in 2% glucose and arrested in G1 by the addition of α factor (1 μg/ml) for 2.5 h. Cells were washed free of α factor, placed in fresh medium containing 2% dextrose, and samples were taken every 15 min. α factor was added back after 90% of the cells had budded. For Figure 4C, strains were grown at 30°C in the presence of 2% galactose and 2% raffinose and arrested in G1 by the addition of α factor (1 μg/ml) for 3 h. 2% dextrose was then added to all strains for an additional 2 h. Cells were washed free of α factor, placed in fresh medium containing 2% dextrose, and samples were taken every 15 min. α factor was added back after 90% of the cells had budded. Protein extracts were prepared by bead-beating in urea lysis buffer. Polyclonal anti-Clb2 antibodies were a generous gift of Doug Kellogg (University of California, Santa Cruz). Anti-Cdk1 and anti-Cdc20 antibodies were from Santa Cruz Biotechnology, Inc. DNA was visualized by DAPI staining and tubulin was visualized by indirect immunofluorescence with antibody YOL1/34 using spheroplasts.

Dissociation Rates

To measure activator dissociation rates, wild-type, Cdc27-A2, Cdc23-A, or Doc1-4A APC/C was immunopurified using IgG-coupled Dynabeads from lysate made by bead-beating a ~1.3 g cell pellet, and incubated with 60 μl of reticulocyte lysate containing in vitro translated ^{35}S-Cdh1 and 400 μl HBST (either with or without 50 μM cyclin) for 1 h at room temperature. After two rapid washes, 1.2 ml reticulocyte lysate containing a 20-fold excess of unlabeled in vitro translated Cdh1 (either with or without 50 μM cyclin) was added as a competitor, so that any ^{35}S-Cdh1 that dissociated would not re-bind. Samples were taken at various times to monitor the amount of bound ^{35}S-Cdh1, and visualized using SDS-PAGE and PhosphorImager. A “pre-mix” control lane, in which labeled and unlabeled Cdh1 are added to the APC/C at the same time, was used to demonstrate that the amount of unlabeled Cdh1 is in excess of the amount of APC/C present. Control experiments also showed that bound ^{35}S-Cdh1 did not decrease when incubated with reticulocyte lysate lacking translated Cdh1 over three

hours. Data points were analyzed using the graphing program Prism and fit to an exponential decay to determine dissociation rate (k_{off}) and $t_{1/2}$ ($t_{1/2}$ =0.69/k_{off}).

To measure substrate dissociation rate, wild-type or Doc1-4A APC/C was immunopurified using IgG-coupled Dynabeads from lysate made by bead-beating a ~2.4 g cell pellet, and incubated with 400 μl in vitro translated Cdh1 and 400 μl HBST for 1 h at room temperature. Unbound activator was removed and the remaining APC/C^{Cdh1} was incubated with 70 μl of ~10 μM ^{125}I-cyclin B for 1 h at room temperature. Unbound ^{125}I-cyclin B was removed and reticulocyte lysate containing an excess of in vitro translated Acm1 (100 μl) was added, so that any ^{125}I-cyclin B that dissociated would not re-bind. Samples were taken at various times to monitor the amount of ^{125}I-cyclin B that remained bound to the APC/C^{Cdh1} on beads.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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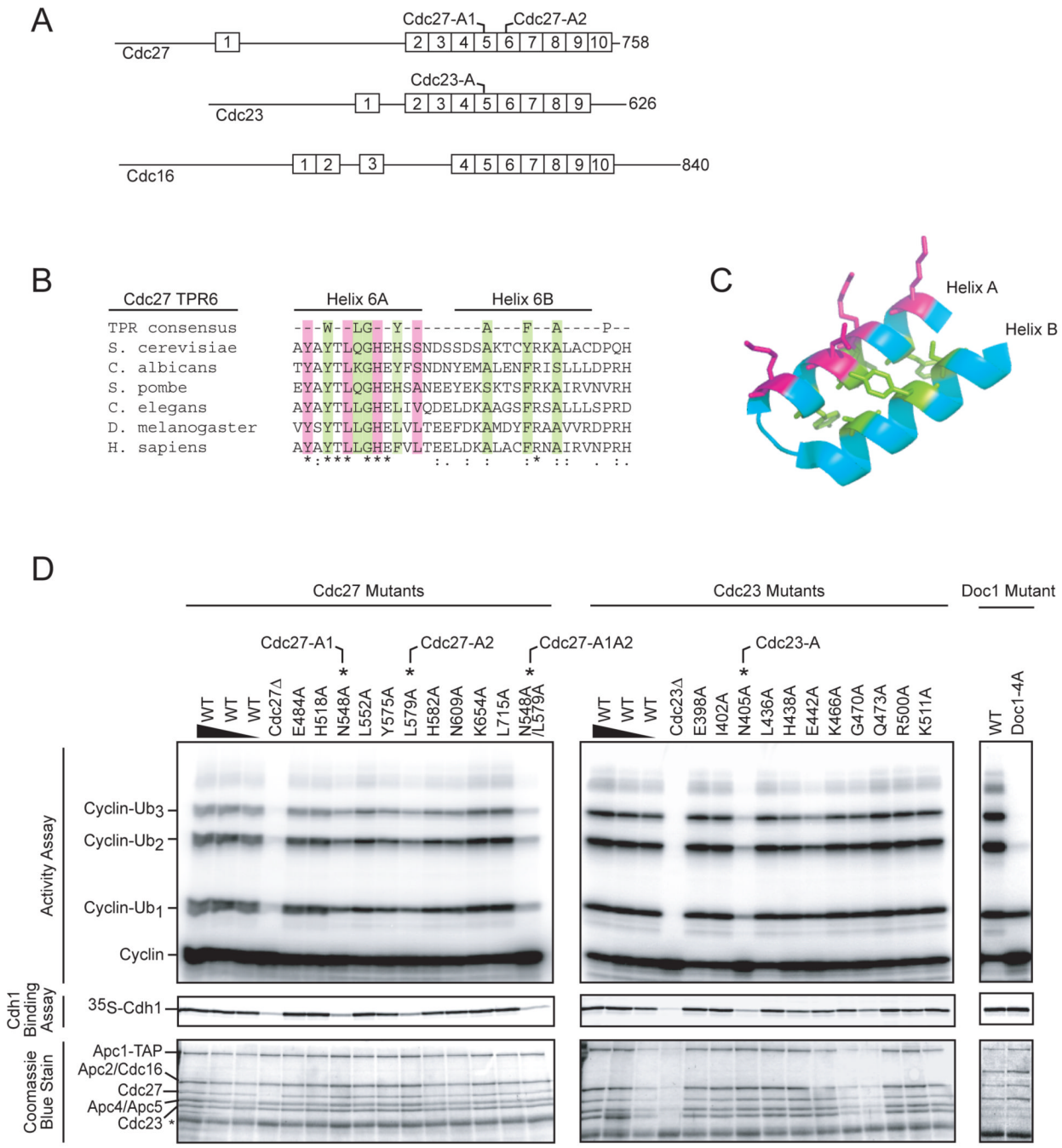

Figure 1. TPR groove mutations in Cdc27 and Cdc23 cause Cdh1 binding defects

(A) The TPRs of Cdc27, Cdc23 and Cdc16 are shown in numbered boxes. Residues that we show are important for activator binding are labeled in TPR 5 and TPR6 of Cdc27 and TPR5 of Cdc23.

(B) A sequence alignment of TPR 6 from Cdc27 homologs illustrates the TPR consensus residues (green) that mediate the interactions between Helix A and Helix B and are distinct from those predicted to form the surface of the TPR groove (pink).

(C) Structure of TPR 3 from the TPR2A domain of Hop (Scheufler et al., 2000), with TPR consensus residues in green and groove-forming residues in pink. This image was generated with MacPyMOL (DeLano, 2007).

(D) Cdc27 and Cdc23 mutants have reduced activator binding. APC/C was immunoprecipitated from strains lacking the wild-type subunit and expressing the indicated Cdc27 or Cdc23 mutant subunit. 10% of the preparation was used to measure ubiquitination activity with ^{125}I-cyclin B (top panels). A limiting amount of Cdh1 was used to normalize for the amounts of immunopurified APC/C. 90% of the immunopurified APC/C was used for measurement of ^{35}S-Cdh1 binding (middle panels). The gel was stained with Coomassie Blue (bottom panels; starred band indicates background IgG from beads) to assess integrity of the APC/C. Three different amounts of wild-type APC/C were tested for comparison (WT lanes). This experiment is representative of three separate experiments, except that the lower amounts of APC/C protein obtained with the Cdc23-K466A and G470A mutants in this experiment were not reproducible. Mutants pursued further are marked with asterisks and labeled. Doc1-4A APC/C was immunopurified using a TAP-tag on Cdc16 rather than Apc1, resulting in the shifted mobility of subunits on the Coomassie Blue-stained gel.

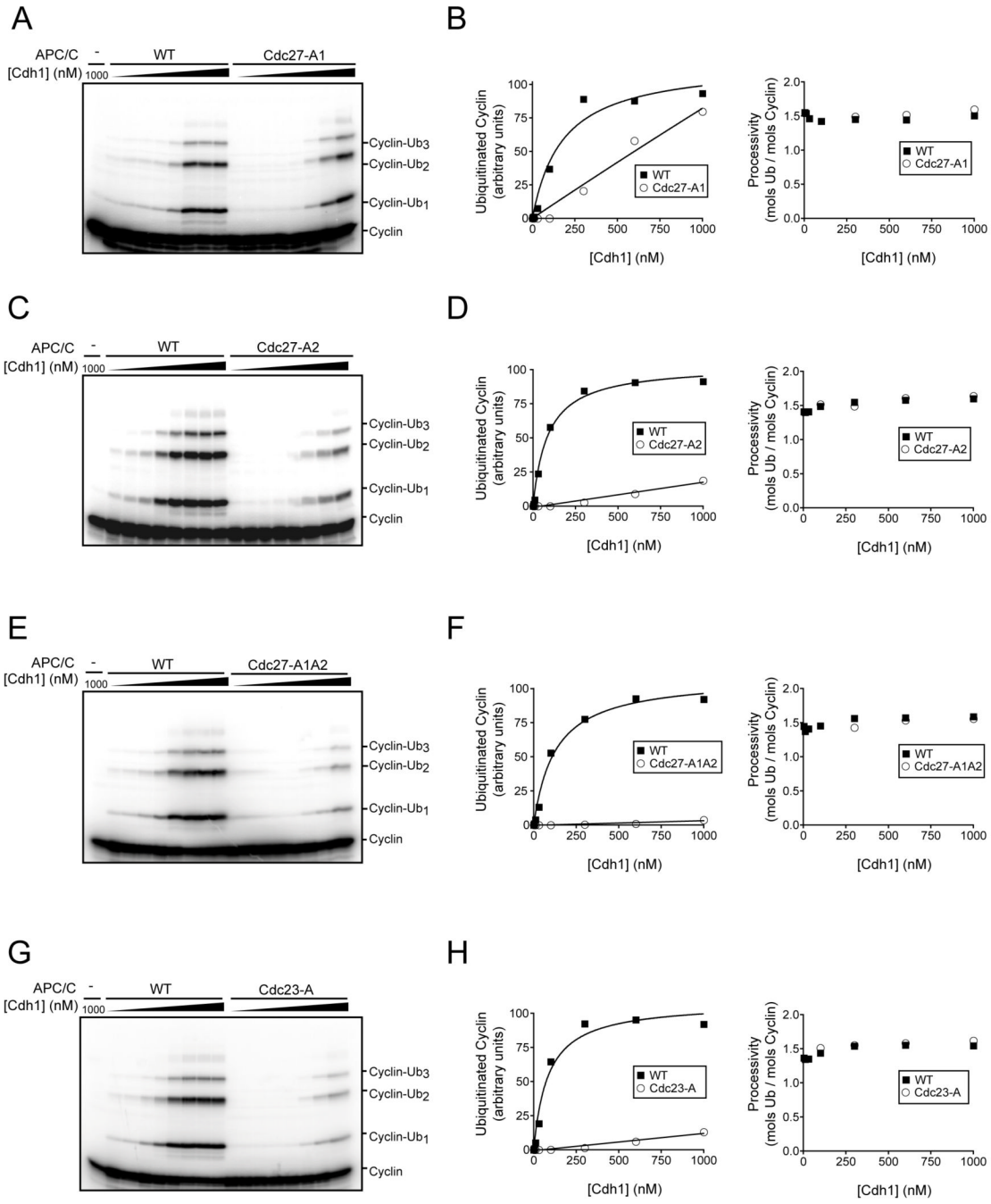

Figure 2. Mutations in Cdc27 and Cdc23 reduce Cdh1 response and activity but not processivity

Recombinant 6xHis-Cdh1, purified from baculovirus-infected insect cells, was titrated into ubiquitination reactions containing E1, E2, ATP, ubiquitin, ^{125}I-cyclin B and either wild-type or mutant TAP-purified APC/C. Cdh1 concentrations were 0, 3, 10, 30, 100, 300, 600, and 1000 nM. After 30 min at room temperature, reaction products were analyzed by SDS-PAGE and PhosphorImager (A, C, E, G). These results are representative of three separate experiments. The amounts of mono- (cyclin B-Ub_1), di- (cyclin B-Ub_2) and tri- (cyclin B-Ub_3) ubiquitinated species were quantified and combined to provide the total amount of ubiquitinated cyclin B. The processivity of ubiquitination was determined by calculating the ratio of ubiquitins to cyclin (B, D, F, H). To observe significant activity with the Cdc27-A1A2

mutant, we used five-fold greater amounts of APC/C, and the quantification reflects the normalized activity per mole of enzyme.

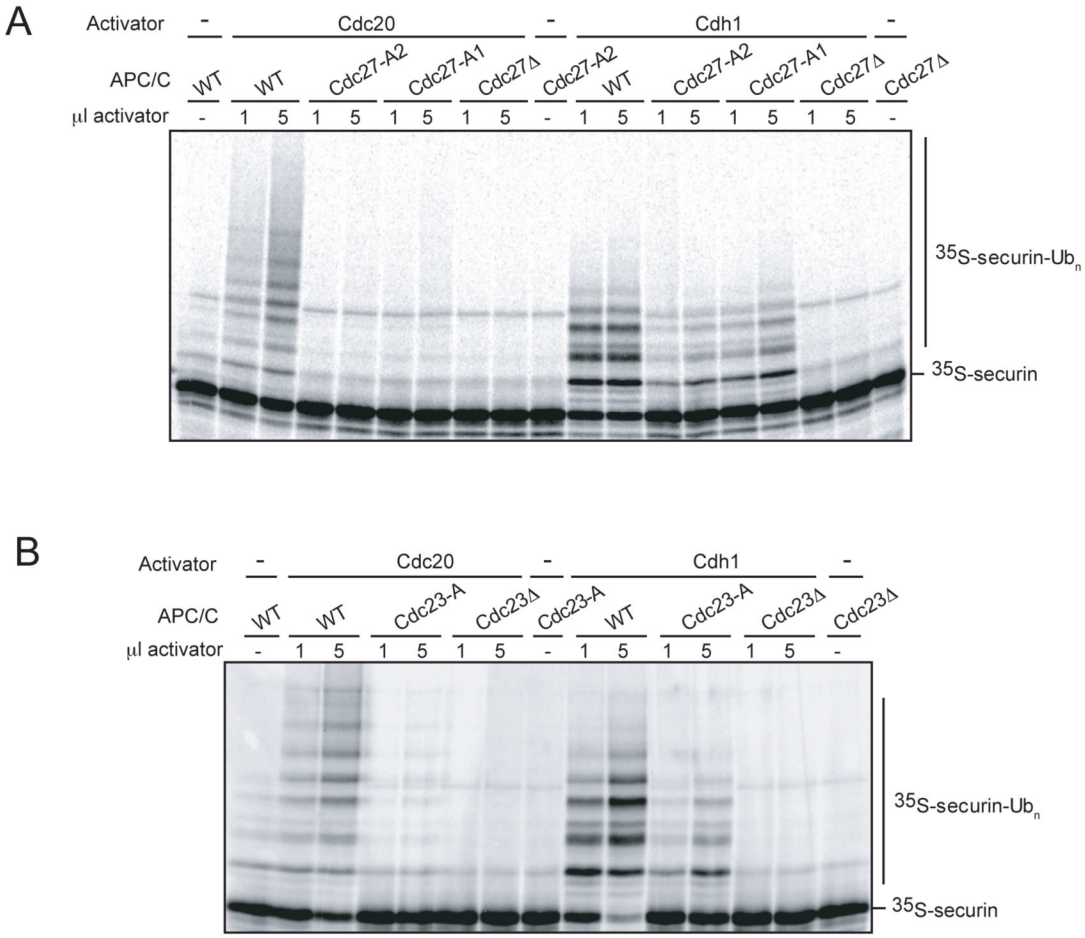

Figure 3. Cdc20-dependent APC/C activity is defective in Cdc27 and Cdc23 mutants in vitro

(A) APC/C reactions were performed with either wild-type, Cdc27-A2, Cdc27-A1, or Cdc27Δ APC/C using ^{35}S-securin as substrate and the indicated amount of in vitro translated, ZZ-tagged Cdh1 or Cdc20 purified from rabbit reticulocyte lysates. Control lanes (-) contained mock activator purifications from 5 μl rabbit reticulocyte lysate without activator. Due to the different levels of activity with Cdc20 and Cdh1, it is not clear whether the mutations had a greater effect on Cdc20-dependent activity than Cdh1-dependent activity.

(B) APC/C reactions were performed with either wild-type, Cdc23-A or Cdc23Δ APC/C as in (A). Note that in the reaction with wild-type APC/C and 5 μl Cdh1, apparent processivity is increased due to substrate depletion.

A

	30°C (cells x10^4 mL^{-1} min^{-1})	37°C (cells x10^4 mL^{-1} min^{-1})
Wild type	2.8	3.4
cdc27-A2	2.8	3.5
cdc27-A1A2	2.9	3.5
cdc23-A	2.7	3.5

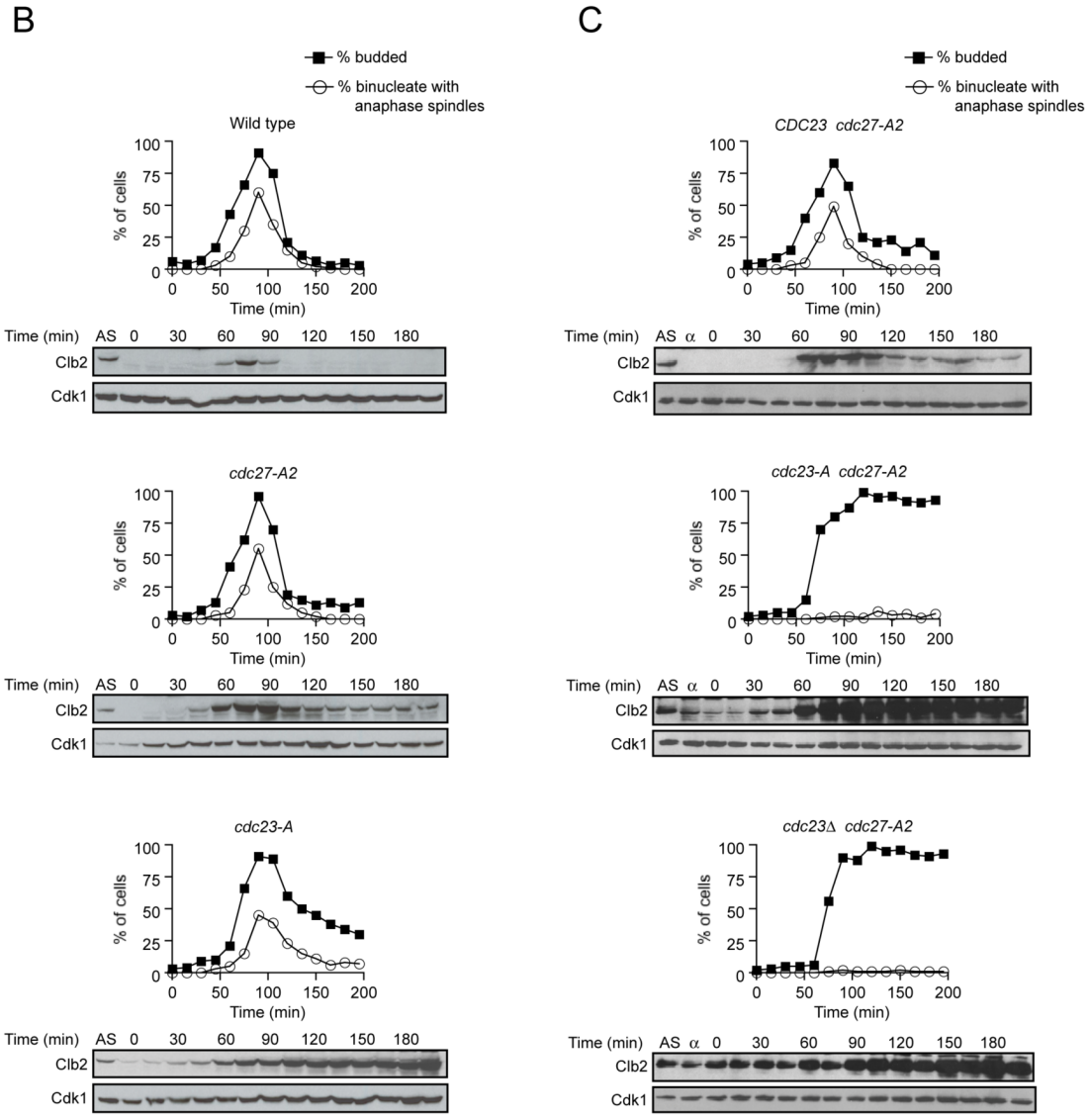

Figure 4. Cdc27 and Cdc23 mutants have mild individual defects in vivo and together are synthetically lethal

(A) Log-phase growth rates, at 30°C and 37°C, of strains in which the indicated mutant gene replaced the endogenous gene. Results are representative of three individual experiments.

(B) Asynchronous cultures of the indicated cells (AS timepoint) were treated with α factor (1 μg/ml) for 2.5 h. α factor was then washed out (zero time point) and cells were harvested at the indicated times. α factor was added back after 90% of the cells were budded. Parallel samples were analyzed directly for budding index (black squares) and fixed, treated with zymolyase, and stained with DAPI to measure chromosome segregation, and with anti-tubulin antibodies to measure spindle elongation. We counted binucleate cells that had elongated

spindles (open circles). Additional samples were prepared for western blotting with anti-Clb2 antibodies and anti-Cdk1 (as a loading control).

(C) Asynchronous cultures of the indicated P_{GAL1}-*CDC23* strains in galactose-containing media (AS timepoint) were treated with α factor for 3 h (α time point), after which dextrose was added for 2 h to turn off expression of wild-type *CDC23*. α factor was washed out (zero time point) and cells released into dextrose-containing media. Samples were taken at the indicated times for analysis of budding index, chromosome segregation, spindle formation, and Clb2 levels as in (B).

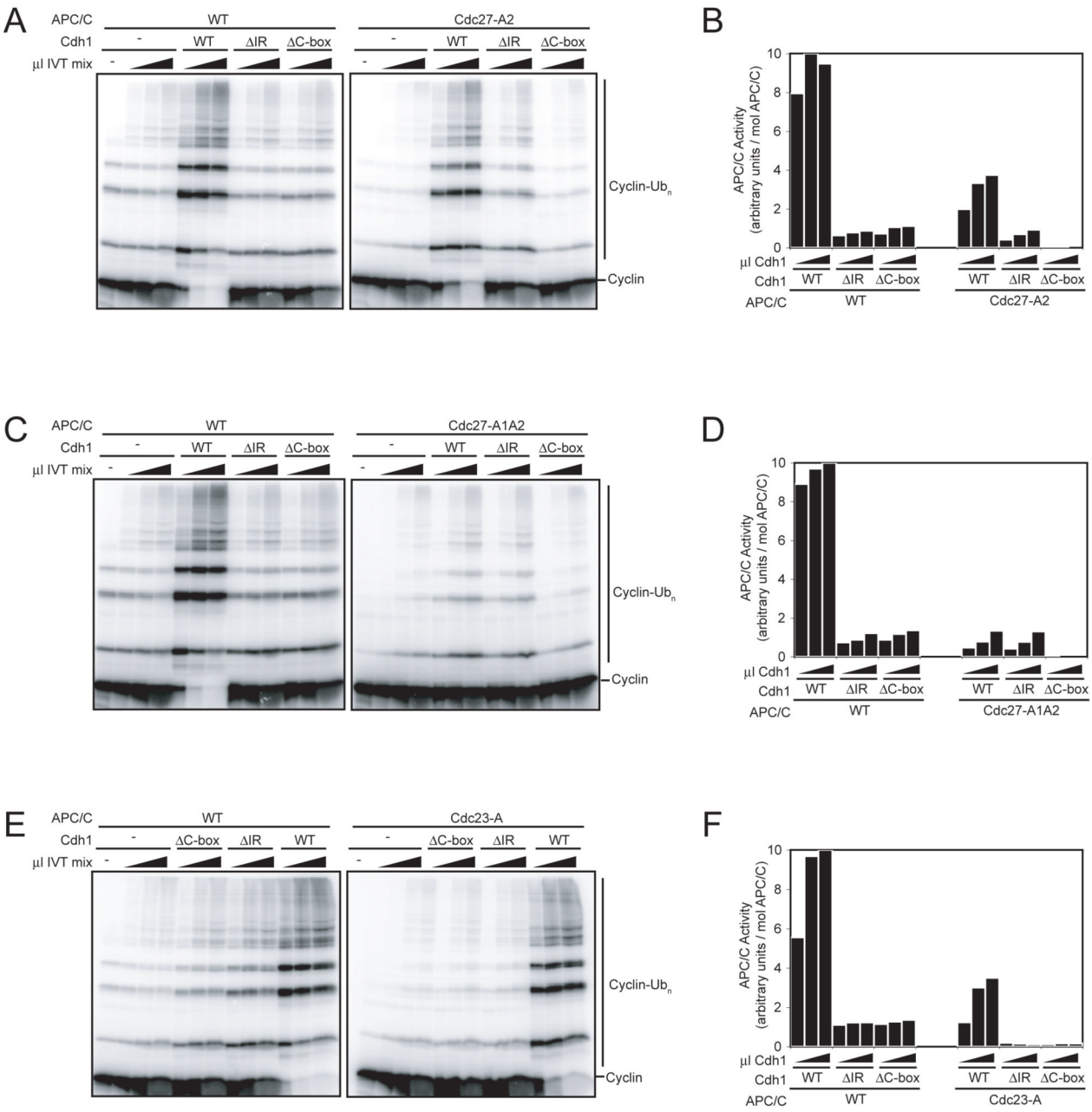

Figure 5. Analysis of activator C-box and IR motif interactions with Cdc27 and Cdc23 mutants

(A, C, E) Reactions containing ^{125}I-cyclin B and either wild-type or Cdc27-A2 APC/C (A), Cdc27-A1A2 APC/C (C), or Cdc23-A APC/C (E) were performed with in vitro translated wild-type, ΔIR (IR to AA), or ΔC-box (IP to AA) Cdh1 in rabbit reticulocyte lysate. An equivalent amount of lysate without activator was added to control reactions (-).

(B, D, F) Quantification of reactions shown in left-hand panels, normalized for background activity (i.e. activity in equivalent (-) lanes was subtracted) and the amount of enzyme added.

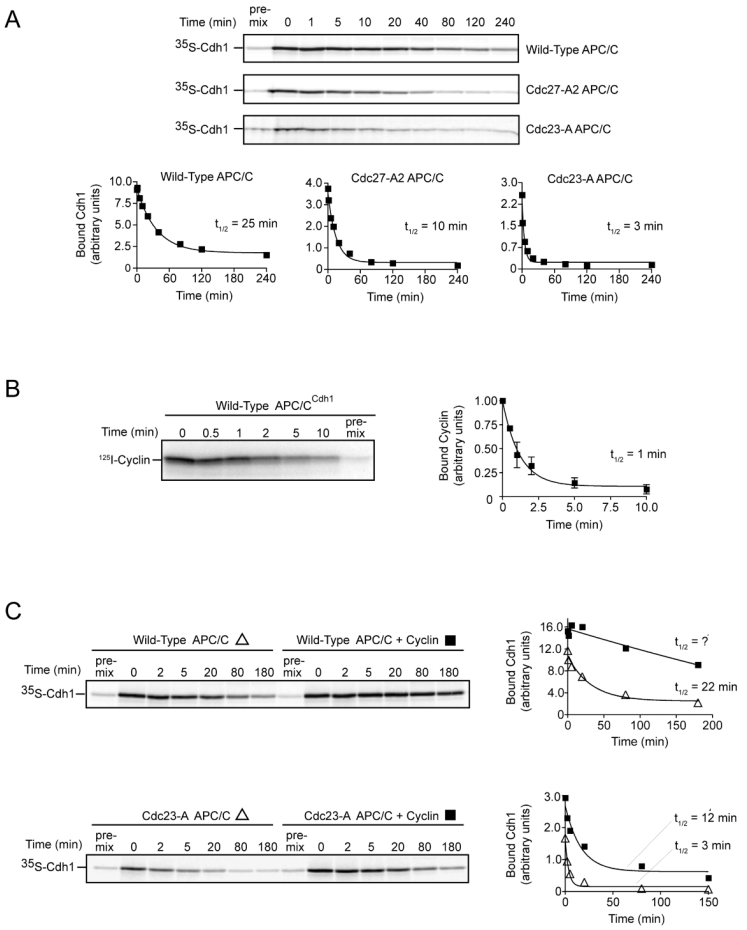

Figure 6. Activator dissociation rate is increased in TPR mutants and decreased in the presence of substrate

(A) Wild-type, Cdc27-A2, or Cdc23-A APC/C was immunopurified and incubated with in vitro translated ^{35}S-Cdh1 for 1 h at room temperature. After two rapid washes, a 20-fold excess of unlabeled in vitro translated Cdh1 was added as a competitor. Samples were taken at the indicated times to monitor the amount of ^{35}S-Cdh1 that remained bound to the APC/C. When ^{35}S-Cdh1 and unlabeled Cdh1 were added to the APC/C at the same time (pre-mix lane), minimal binding of ^{35}S-Cdh1 was observed. Coomassie staining indicated that the same amount of APC/C was present in each lane.

(B) Wild-type APC/C was immunopurified and incubated with in vitro translated Cdh1 for 1 h at room temperature. Unbound activator was removed and the remaining APC/C^{Cdh1} was incubated with ^{125}I-cyclin B for 1 h at room temperature. Unbound ^{125}I-cyclin B was removed and an excess of in vitro translated Acm1 (Enquist-Newman et al., 2008) was added as substrate competitor. Samples were taken at the indicated times to monitor the amount of ^{125}I-cyclin B that remained bound to APC/C^{Cdh1}. When ^{125}I-cyclin B and Acm1 were added to the APC/C at the same time (pre-mix lane), minimal binding of ^{125}I-cyclin B was observed. Coomassie staining indicated that the same amount of APC/C was present in each lane. A representative experiment is shown on the left, and the graph on the right shows the average of values from three separate experiments (+/- SEM).

(C) Dissociation of Cdh1 from wild-type or Cdc23-A APC/C was measured as in panel (A), except that 50 μM cyclin was included as indicated during Cdh1 binding and dissociation. Coomassie staining indicated that the same amount of APC/C was present in each lane.

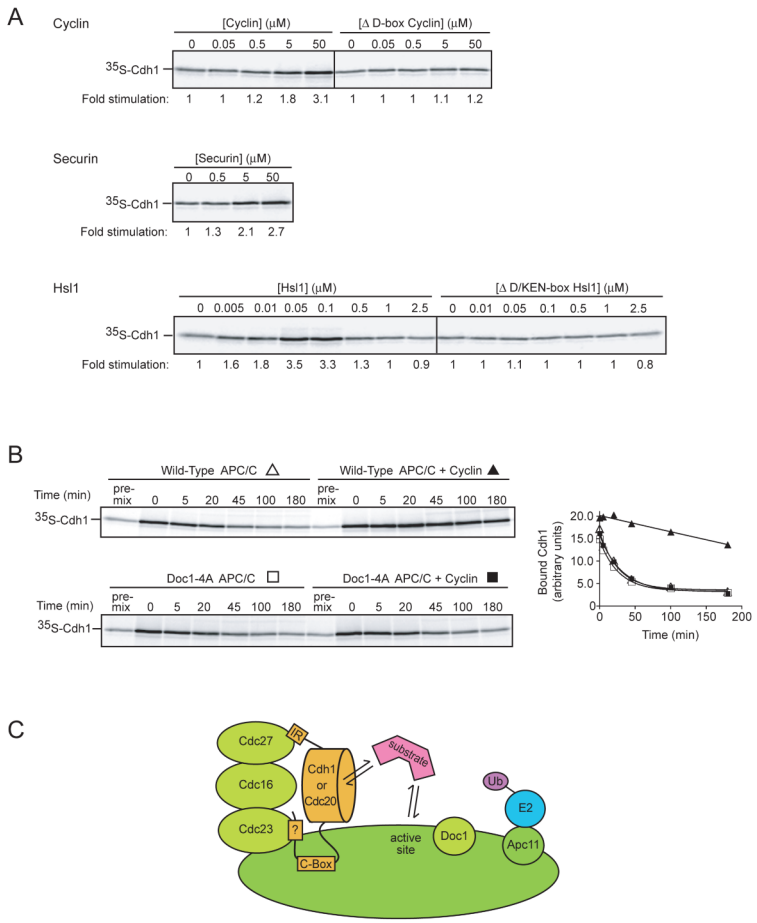

Figure 7. Substrate promotes activator binding through a bivalent bridging interaction

(A) Cdc23-A APC/C was immunopurified and incubated for 1 h at room temperature with in vitro translated ^{35}S-Cdh1 and the indicated concentrations of purified cyclin, securin, or Hsl1 fragments. Immunoprecipitates were washed two times for 30 s each before analysis by SDS-PAGE. D-box mutant cyclin and D/KEN-box mutant Hsl1 (Burton and Solomon, 2001) were also tested. Fold stimulation is the ratio of the amount of ^{35}S-Cdh1 bound in the presence of substrate to the amount bound with no substrate.

(B) Dissociation of Cdh1 from wild-type or Doc1-4A APC/C was measured in the absence and presence of 50 μM cyclin as in Figure 6C. Coomassie staining indicated that the same amount of APC/C was present in each lane. Half-times of Cdh1 dissociation were as follows: wild-type APC/C: 20 min; wild-type APC/C plus cyclin: >200 min; Doc1-4A APC/C: 20 min; Doc1-4A APC/C plus cyclin: 22 min.

(C) We propose that activator binds the APC/C at sites on Cdc27 and Cdc23 and shares substrates with a site on the APC/C core during catalysis. Our results suggest that Cdc27 interacts with the IR motif of Cdh1 and Cdc23 interacts with an unknown site on the activator. The bivalent substrate-binding model is explored in detail in Figure S10.