Role of ubiquitylation of components of mitotic checkpoint complex in their dissociation from anaphase-promoting complex/cyclosome

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The mitotic checkpoint system ensures the fidelity of chromosome segregation in mitosis by preventing premature initiation of anaphase until correct bipolar attachment of chromosomes to the mitotic spindle is reached. It promotes the assembly of a mitotic checkpoint complex (MCC), composed of BubR1, Bub3, Cdc20, and Mad2, which inhibits the activity of the anaphase-promoting complex/cyclosome (APC/C) ubiquitin ligase. When the checkpoint is satisfied, anaphase is initiated by the disassembly of MCC. Previous studies indicated that the dissociation of APC/C-bound MCC requires ubiquitylation and suggested that the target of ubiquitylation is the Cdc20 component of MCC. However, it remained unknown how ubiquitylation causes the release of MCC from APC/C and its disassembly and whether ubiquitylation of additional proteins is involved in this process. We find that ubiquitylation causes the dissociation of BubR1 from Cdc20 in MCC and suggest that this may lead to the release of MCC components from APC/C. BubR1 in MCC is ubiquitylated by APC/C, although to a lesser degree than Cdc20. The extent of BubR1 ubiquitylation was markedly increased in recombinant MCC that contained a lysine-less mutant of Cdc20. Mutation of lysine residues to arginines in the N-terminal region of BubR1 partially inhibited its ubiquitylation and slowed down the release of MCC from APC/C, provided that Cdc20 ubiquitylation was also blocked. It is suggested that ubiquitylation of both Cdc20 and BubR1 may be involved in their dissociation from each other and in the release of MCC components from APC/C.

cell cycle | ubiquitin | protein degradation

The mitotic checkpoint is a surveillance system that ensures the fidelity of chromosome segregation in mitosis. It senses the existence of kinetochores that are not attached correctly to the mitotic spindle and prevents premature initiation of anaphase by the inhibition of anaphase-promoting complex/cyclosome (APC/C) ubiquitin ligase. APC/C is inhibited by the mitotic checkpoint complex (MCC), which is assembled from its components (BubR1, Bub3, Cdc20, and Mad2) when the checkpoint is turned on. When the checkpoint is turned off, MCC is disassembled and APC/C regains activity and targets for degradation-specific cell cycle regulatory proteins such as securin, an inhibitor of anaphase initiation (reviewed in refs. 1–5).

We have been studying pathways of disassembly of the MCC, necessary for inactivation of the mitotic checkpoint. Our previous studies indicated that MCC exists in free and in APC/C-bound pools and that disassembly of MCC in both pools is required to release APC/C from checkpoint arrest (6). The disassembly of MCC in both pools requires ATP (7), but their pathways are different (6, 7). Thus, in the case of free MCC, Mad2 is released by the joint action of the Mad2-binding protein p31^{comet} and the AAA-ATPase TRIP13 (8, 9), and Cdc20 is dissociated from BubR1 either by the action of CCT chaperonin (10) or by p31^{comet}-stimulated phosphorylation of Cdc20 (11). By a very different mechanism, the disassembly of APC/C-bound MCC requires APC/C-catalyzed ubiquitylation (6, 7, 11). Several lines of evidence suggested that

the Cdc20 component in MCC is a major target of this ubiquitylation. Pan and Chen (12) reported that in *Saccharomyces cerevisiae* the mitotic checkpoint causes rapid degradation of Cdc20, a process that requires its association with Mad2 and functional APC/C. Further studies have shown that this process is conserved in mammalian cells (13, 14). It was also reported that in an in vitro system that lacked proteasomes, ubiquitylation caused release of all MCC components from APC/C (11). It thus seems that ubiquitylation is sufficient to release MCC components from APC/C, even without subsequent degradation by the proteasome. In that study, marked accumulation of polyubiquitylated Cdc20 was observed (11). More recently, several investigators reported that APC15, a subunit of the APC/C, is required for Cdc20 ubiquitylation and for MCC release from APC/C (15–17). It has been suggested that APC15 stabilizes APC/C in a conformation that positions its catalytic site in proximity to lysine acceptor sites of Cdc20 in MCC (18, 19). Thus, there is a large body of evidence (although still only circumstantial) for a role of Cdc20 ubiquitylation in MCC release. It is not known, however, whether Cdc20 is the only target of ubiquitylation. It is also not known how ubiquitylation causes MCC release. It has been suggested that ubiquitylation of Cdc20 causes its dissociation from Mad2 (11), but subsequently we found that ubiquitylated Cdc20 released from APC/C is still bound to Mad2 (6).

We now report that ubiquitylation causes dissociation of BubR1 from Cdc20 and thus may lead to the release of MCC components from APC/C. We found that BubR1 in MCC is also

Significance

The mitotic checkpoint system is important for ensuring the correct segregation of chromosomes in mitosis. When chromosomes are not attached correctly to the mitotic spindle, a mitotic checkpoint complex (MCC) is formed that prevents chromosome separation by inhibiting the action of the anaphase-promoting complex/cyclosome (APC/C) ubiquitin ligase. MCC is disassembled when the checkpoint is turned off. The disassembly of APC/C-bound MCC was known to require ubiquitylation, but the mechanisms of this process remained unknown. Here we show that two different components of MCC may be ubiquitylated and suggest that these ubiquitylation events lead to their dissociation from each other and from APC/C. Thus, this investigation provides insight into the molecular events responsible for the inactivation of the mitotic checkpoint.

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ubiquitylated by APC/C, although to a lesser extent than Cdc20. The extent of BubR1 ubiquitylation is markedly increased in recombinant MCC that contains a lysine-less mutant of Cdc20. Partial blocking of BubR1 ubiquitylation by mutating lysine residues in its N-terminal region slows down the release of MCC from APC/C, provided that Cdc20 ubiquitylation is also blocked. We suggest that ubiquitylation of both Cdc20 and BubR1 contribute to the disassembly of APC/C-bound MCC.

Results

Ubiquitylation and Release of APC/C-Bound MCC Components in a Preparation from Checkpoint-Arrested HeLa Cells. It has been shown previously that APC/C-promoted ubiquitylation is required for the release of MCC bound to APC/C but not for the disassembly of free MCC (6, 7). Some further characteristics of the release of MCC from APC/C are shown in Fig. 1A. In this experiment, APC/C-MCC from checkpoint-arrested HeLa cells was isolated by immunoprecipitation with anti-Cdc27, immunoprecipitates were incubated with the indicated additions, and the amounts of MCC components that remained bound to APC/C were estimated by immunoblotting (see *Materials and Methods*). In accordance with previous results (7, 11), considerable release of MCC components could be observed following incubation with an ubiquitylation mixture (consisting of E1, UBCH10, ATP, and ubiquitin) (lane 2). By contrast, incubation with TRIP13 and p31^{comet}, which release Mad2 from free MCC (8), had no such influence on APC/C-bound MCC (Fig. 1A, lane 3). Also in contrast to the characteristics of other ATP-dependent processes involved in the disassembly of free MCC, we found that in the ubiquitylation-stimulated release of MCC from APC/C, ATP could be effectively replaced by its β - γ nonhydrolyzable analog adenosine 5'-(β , γ)-imidotriphosphate (AMP-PNP, lane 5). Actions of CCT chaperonin require hydrolysis of the β - γ bond of ATP (20), while E1-dependent ubiquitylation involves hydrolysis of its α - β bond (21). Thus, our results ruled out the possibility that, in addition to ubiquitylation, residual CCT chaperonin-promoted MCC disassembly (10) may also be involved in this process.

We have previously observed significant ubiquitylation of the BubR1 component of MCC (6). The characteristics of the ubiquitylation and release from APC/C of BubR1 and of Cdc20 were further examined in the experiments shown in Fig. 1B and C. Following incubation of APC/C-MCC with the ubiquitylation mixture, there was considerable accumulation of ubiquitylated derivatives of Cdc20 in the total reaction mixture (Fig. 1B, lanes 2 and 3). BubR1 was also ubiquitylated, but to a lesser extent (Fig. 1C, lanes 2 and 3). In different experiments, the extent of ubiquitylation was in the range of 40–70% for Cdc20 and of 10–20% for BubR1. With both Cdc20 and BubR1, most of the ubiquitylated derivatives accumulated in high-molecular-size polyubiquitylated forms.

To examine the relationship between ubiquitylation and release of these MCC components, we also looked for the presence of ubiquitylated components that remained bound to APC/C (anti-Cdc27 immunoprecipitates) and those released from APC/C (supernatant fraction). Following the ubiquitylation reaction, mainly free MCC components remained bound to APC/C, with very small amounts of polyubiquitylated high-molecular-weight Cdc20 and BubR1 (Fig. 1B, lanes 5 and 6). Correspondingly, most polyubiquitylated derivatives of Cdc20 (Fig. 1B, lanes 8 and 9) and of BubR1 (Fig. 1C, lanes 5 and 6) were in the fraction released from APC/C. In addition, BubR1 was also released in lower-molecular-weight forms, which may be a mixture of free BubR1 and of BubR1 ligated to a few molecules of ubiquitin (Fig. 1C, lanes 5 and 6). These results indicate that polyubiquitylation of both Cdc20 and of BubR1 from MCC is accompanied by their release from APC/C.

Cdc20 and BubR1 Released from APC/C Are Dissociated from Each Other. We next examined whether polyubiquitylation of Cdc20 and BubR1 is accompanied not only by the release of MCC from APC/C but

Fig. 1. Ubiquitylation and release of APC/C-bound MCC components from checkpoint-arrested HeLa cells. (A) Characteristics of the release of MCC from APC/C. APC/C-MCC was immunopurified from extracts of checkpoint-arrested cells and was incubated (23 °C, 60 min) as described in *Materials and Methods*, with additions as specified. Where indicated, ATP (1 mM) was added together with 10 mM phosphocreatine and 100 mg/mL creatine phosphokinase. AMP-PNP was supplemented at 2 mM. Recombinant TRIP13 and p31^{comet} (8) were added at 100 nM and 50 nM, respectively. Following incubation, anti-Cdc27 beads were washed and MCC components that remained associated with APC/C were estimated by immunoblotting (see *Materials and Methods*). (B) Ubiquitylated Cdc20 is released from APC/C. A preparation of APC/C-MCC bound to anti-Cdc27 beads was incubated with or without the ubiquitylation system as in A, and then reaction products were processed to either an unfractionated mixture ("Total", Left), washed beads ("Bound to APC/C", lanes 4–6, Center), or supernatant ("Released from APC/C", Right), as described in *Materials and Methods*. These fractions were subjected to immunoblotting with either anti-Cdc20 (Left and Right) or with a mixture of anti-Cdc20 and anti-BubR1 antibodies (Center). Electrophoretic migration positions of molecular-size marker proteins (kDa) are indicated on the right side. (C) Ubiquitylated BubR1 is released from APC/C. The experiment was similar to that described in B, except that all samples were immunoblotted for BubR1. AMP-PNP, adenosine 5'-(β , γ -imido)triphosphate; Syst., system; Ub, ubiquitylation; Ub(n), multiple ubiquitin molecules.

also by their dissociation from each other. For this purpose, material released from APC/C-MCC immunoprecipitates was subjected to a second immunoprecipitation with either anti-Cdc20 or anti-BubR1 antibodies. The second immunoprecipitates ("Re-IP") were immunoblotted for either Cdc20 (Fig. 2A) or for BubR1 (Fig. 2B). In parallel controls, immunoprecipitation of recombinant MCC by the same antibodies was carried out. As expected, Cdc20 in the MCC control was precipitated not only by anti-Cdc20 (Fig. 2A, lane 4) but also by anti-BubR1 (Fig. 2A, lane 3), indicating the association of Cdc20 with BubR1 in MCC. Another control precipitation by nonimmune IgG (lane 2) showed that the amount of nonspecific binding was very small. When polyubiquitylated Cdc20, released from APC/C, was subjected to a similar reimmunoprecipitation procedure, it was precipitated only by anti-Cdc20 antibody (lane 8) but not by anti-BubR1 (lane 7). This indicates that released derivatives of Cdc20 and BubR1 are separated from each other. This is different from the case of Mad2, which is released from APC/C-bound MCC still bound to polyubiquitylated Cdc20 (6).

To substantiate the above conclusion, we subjected similar reimmunoprecipitates to immunoblotting for BubR1 (Fig. 2B). In this case, too, BubR1 of the recombinant MCC control was immunoprecipitated by either anti-BubR1 (Fig. 2B, lane 3) or by anti-Cdc20 (lane 4), indicating the validity of the procedure. By contrast, derivatives of BubR1 released from APC/C were precipitated only by anti-BubR1 (lane 7) but not by anti-Cdc20 (lane 8), showing again the dissociation of BubR1 derivatives from those of Cdc20 in material released from APC/C. In the latter case, only a very small amount of released material, which corresponded in size to free BubR1, was precipitated with anti-Cdc20 (lane 8). These results, indicating ubiquitylation-induced dissociation of Cdc20 and BubR1 in MCC to forms that have low affinity for APC/C, may suggest that Cdc20-BubR1 dissociation is the primary event that leads to the release of MCC components from APC/C (see *Discussion*).

The experiments described above indicated some resemblance between the characteristics of ubiquitylation-dependent release of Cdc20 and of BubR1 from APC/C-MCC: in both cases, polyubiquitylated derivatives were almost entirely released from APC/C and in this process they were also dissociated from each other. These observations raised the possibility that ubiquitylation of BubR1, as that of Cdc20, may be involved in the release of MCC components from APC/C. The experiments described below were designed to examine this question.

Ubiquitylation-Dependent Disassembly of Recombinant MCC Containing a K-less Cdc20 Mutant. To gain more insight into the roles of ubiquitylation of different MCC components in MCC disassembly, we used recombinant MCCs containing suitable mutants. We first took advantage of the availability of a mutant of Cdc20 in which all lysine residues were converted to arginines (“Cdc20^{K-less}”, ref. 13). It has been shown previously that Cdc20^{K-less} is released from MCC in checkpoint-arrested cells (13, 15), and it has been proposed that this may be due to the ubiquitylation of some other target protein (15). However, this problem has not yet been studied *in vitro*, and it has not been examined whether the disassembly of Cdc20^{K-less}-containing MCC actually requires ubiquitylation. In the experiment shown in Fig. 34, we examined this problem *in vitro* by the binding of MCCs containing WT or K-less Cdc20 to APC/C purified from HeLa cells and then testing the effect of the ubiquitylation system on the release of these MCC complexes from APC/C. Components of WT recombinant MCC were released from APC/C in a ubiquitylation-dependent manner, indicating that the characteristics of this process are similar to those of “native” MCC from HeLa cells. The release from APC/C of all components of Cdc20^{K-less}-containing MCC also required the ubiquitylation system and occurred at a rate only slightly slower than that of WT MCC (Fig. 34).

We examined several possible explanations for the dependence on ubiquitylation of the disassembly of Cdc20^{K-less}-containing MCC. One possibility was that K-less Cdc20 undergoes unusual ubiquitylations, such as those on the N terminus (22) or on Ser/Thr residues (23). As shown in Fig. 3B, lanes 8–10, some “noncanonical” ubiquitylation of Cdc20^{K-less} was detected, but its extent was very low under the experimental conditions employed in these experiments. This suggested that the ubiquitylation of some other target protein may have caused the release of components of Cdc20^{K-less}-containing MCC.

We also examined the possibility that the ubiquitylation of some components of APC/C may decrease its affinity to MCC and may thus lead to MCC release. To test this notion, we subjected purified APC/C from mitotic *Xenopus* extracts (that lack endogenous MCC) to an ubiquitylation reaction in the absence of MCC, and following washing that removed the ubiquitylation system the binding of MCC to APC/C was tested. As shown in [Fig. S1, Left](#), polyubiquitylation of some APC/C (or APC/C-associated) proteins was detected following incubation with the ubiquitylation system. However, we could not detect any difference between the extent of subsequent binding of MCC to ubiquitylated vs. unmodified APC/C ([Fig. S1, Right](#)).

Fig. 2. Ubiquitylation causes the dissociation of BubR1 from Cdc20. (A) Cdc20 released from APC/C is dissociated from BubR1. A preparation of APC/C-MCC from checkpoint-arrested HeLa cells bound to anti-Cdc27 beads was incubated with the ubiquitylation system and supernatants were collected as described in *Materials and Methods*. Samples of 60 μ L supernatants were subjected to reimmunoprecipitation with 5 μ g either nonimmune rabbit IgG (Pierce) or affinity-purified rabbit antibodies directed against human Cdc20 or BubR1. Samples of recombinant WT MCC (1 nM) were treated similarly. Following incubation at 4 °C for 2 h, samples were mixed with protein A beads (10 μ L, packed volume), rotated at 4 °C for 2 h, and then washed four times with a solution containing 50 mM Tris-HCl (pH 7.2), 0.3 M NaCl, 1% Nonidet P-40, and 1 mg/mL BSA. Samples of precipitates were separated on SDS-polyacrylamide gels and immunoblotted for Cdc20. Inputs are samples of supernatant before reimmunoprecipitation. (B) Samples described in A were immunoblotted for BubR1. IB, immunoblotting; Re-IP, reimmunoprecipitation; Ub, ubiquitylation.

Fig. 4. Influence of 5-304 K-less mutations in BubR1 on its ubiquitylation and on the release of MCC components from APC/C. (A) Ubiquitylation of BubR1^{5-304 K-less}. Recombinant MCCs, composed of the indicated proteins which also contained K-less Cdc20, were bound to APC/C^{Cdc20} from extracts of mitotic *Xenopus* eggs (see *Materials and Methods*) and then were incubated with or without the ubiquitylation mixture. Samples of the total reaction mixture were immunoblotted for BubR1. (B) Influence of 5-304 K-less mutation of BubR1 on disassembly from APC/C of MCCs that also contained K-less Cdc20. Recombinant MCCs that contained either WT BubR1 and K-less Cdc20 (circles, blue lines) or 5-304 K-less BubR1 and K-less Cdc20 (squares, red lines), were bound to APC/C^{Cdc20} from mitotic *Xenopus* egg extracts, and the ubiquitylation-dependent release of MCC components from APC/C was determined as described in *Materials and Methods*. Immunoblot of the experiment (in duplicates) is shown in Fig. S2, and its quantitation (mean of duplicates) in B. (C) Proposed events in ubiquitylation-dependent dissociation of MCC components from APC/C. The ubiquitylation of either Cdc20 or of BubR1 in MCC causes, by an as-yet-unknown mechanism, their dissociation from each other and the release of all dissociated components from APC/C. All released components have low affinities for APC/C. Mad2 is not dissociated from Cdc20 in this process and is possibly released subsequently by the joint action of TRIP13 and p31^{comet} (8). Bub3 is constitutively bound to BubR1 and is not shown in this diagram. Ub, ubiquitylation.

BubR1 and K-less Cdc20, although release was not arrested completely (Fig. 4B and Fig. S2). The partial effect on MCC release may be due to incomplete blocking of BubR1 ubiquitylation by the 5-304 K-less mutation. These data suggested the possible involvement of both Cdc20 and BubR1 ubiquitylation in MCC release (see *Discussion*).

Discussion

Previous studies indicated that APC/C-catalyzed ubiquitylation is required for the release of MCC from APC/C (6, 11–14), but it remained unclear which proteins are the targets of ubiquitylation and what is the mechanism by which ubiquitylation promotes this process. In agreement with previous findings (6), we observed that incubation of APC/C-MCC with the ubiquitylation system caused marked polyubiquitylation of Cdc20 and to a lesser extent of BubR1, and furthermore found that essentially all polyubiquitylated Cdc20 and BubR1 were released from APC/C (Fig. 1 *B* and *C*). This may indicate either that polyubiquitylation of MCC components decreases the binding of assembled MCC to APC/C or that it dissociates MCC into components that have decreased affinity for APC/C. The second possibility appears to be correct, since our reimmunoprecipitation experiments indicated that ubiquitylation did not release intact MCC from APC/C, but rather, dissociated BubR1 from Cdc20 (Fig. 2). This outcome is different from the case of Mad2, which is released from APC/C-MCC still bound to ubiquitylated Cdc20 (6). Since free BubR1 and Cdc20-Mad2 subcomplex have very low affinities to APC/C, compared with that of MCC (24), it is possible that the primary effect of ubiquitylation is to dissociate BubR1 from Cdc20 in MCC, which is followed by secondary release of all dissociated MCC components from APC/C.

The above experiments, which showed a resemblance in properties of the release of polyubiquitylated Cdc20 and BubR1 from APC/C, raised the question of whether polyubiquitylation of BubR1 may also contribute to the release of MCC components from APC/C. To examine this question, we first used recombinant MCC that contained a K-less Cdc20 mutant. Our *in vitro* experiments confirmed previous *in vivo* results indicating that this mutant is effectively released from APC/C (15). We furthermore showed that the disassembly of Cdc20^{K-less}-containing MCC is also ubiquitylation dependent (Fig. 3*A*). This did not seem to be due to the ubiquitylation of K-less Cdc20 on nonlysine residues, which was very low under the employed experimental conditions (Fig. 3*B*), or to the ubiquitylation of APC/C (Fig. S1). Notably, we found that the polyubiquitylation of BubR1 was markedly increased in MCC that contained the K-less mutant of Cdc20 (Fig. 3*C* and *D*). Thus, the lack of ubiquitylation acceptor sites on Cdc20 apparently diverts a larger proportion of ubiquitylation activity to BubR1 in MCC. This, in turn, may increase the contribution of BubR1 ubiquitylation in MCC disassembly.

To further examine the possible role of BubR1 ubiquitylation in the disassembly of APC/C-bound MCC, we tried to block BubR1 ubiquitylation by mutating its lysine residues to arginines. We were limited in this to mutations in the N-terminal portion of BubR1 (5-304), since further mutations interfered with the assembly of BubR1 to MCC. This mutation in BubR1 decreased partially its ubiquitylation in MCC (Fig. 4A and Fig. S3A). The 5-304 K-less BubR1 mutation also decreased the rate of disassembly of MCC components from APC/C, provided that Cdc20 ubiquitylation was also blocked (Fig. 4B and Figs. S2 and S3). These data are consistent with the interpretation that the ubiquitylation of both Cdc20 and BubR1 plays a role in the disassembly of APC/C-bound MCC. When Cdc20 is WT, its ubiquitylation is sufficient for disassembly and there is less requirement for BubR1 ubiquitylation for release from APC/C. However, with K-less Cdc20 in MCC, a requirement for ubiquitylation of BubR1 was more pronounced due to lack of ubiquitylation of Cdc20.

The present results suggest that ubiquitylation-dependent dissociation of BubR1 from Cdc20 in MCC may be the primary event that leads to the release of MCC components from APC/C. We also show that ubiquitylation of both Cdc20 and (to a lesser extent) of BubR1 may contribute to this process. However, it is still not clear how ubiquitylation causes BubR1–Cdc20 dissociation. While in intact cells polyubiquitylation of Cdc20 is followed by its proteasomal degradation (12–14, 25), in purified systems that lack proteasomes, ubiquitylation still causes disassembly of APC/C-bound MCC (refs. 6 and 11 and this study). This suggests that even in vivo, disassembly may precede proteolysis. Further studies are needed to elucidate this problem. It also remains unknown what is the signal that triggers the ubiquitylation of Cdc20 and BubR1, how is the signal transmitted to the APC/C, and whether this process is regulated by the state of the mitotic checkpoint system.

Fig. 4C summarizes our proposal on events that take place in the ubiquitylation-dependent dissociation of MCC components from APC/C. According to this model, when either Cdc20 or BubR1 cannot be ubiquitylated, the ubiquitylation of the other component (i.e., of either BubR1 or Cdc20, respectively) becomes essential for the dissociation of MCC from APC/C. It appears reasonable to assume that, in either case, the continued association of MCC with APC/C prevents the release of APC/C activity from checkpoint inhibition and thus inhibits the degradation of cyclin B1 and securin as well as exit from mitosis.

Materials and Methods

To examine the disassembly of APC/C-bound MCC, we have used three different preparations, in all of which disassembly absolutely required ubiquitylation. In the first, “natural” MCC was isolated bound to APC/C from extracts of checkpoint-arrested HeLa cells by immunopurification with anti-Cdc27 beads. Such

preparations were used in the experiments described in Figs. 1 and 2. For experiments that required the use of recombinant mutant MCCs, we needed preparations of APC/C devoid of endogenous MCC. For this purpose, we used preparations of APC/C that were purified from HeLa cells by affinity chromatography on p13^{SUC1}-Sepharose (26), from which residual MCC was further depleted by immunodepletion with α -BubR1 (experiments in Fig. 3). We have also used APC/C^{Cdc20} purified from extracts of mitotic *Xenopus* eggs, which are completely devoid of endogenous MCC (experiments in Fig. 4). Experimental details on these three preparations are provided in *SI Materials and Methods*.

In all of the preparations, samples of APC/C bound to α -Cdc27 beads, to which appropriate MCCs were bound, were incubated with a reaction mixture that contained in a volume of 40 μ L: 40 mM Tris-HCl (pH 7.6), 5 mM MgCl₂, 1 mM DTT, 10% (vol/vol) glycerol, 2 mg/mL BSA, and 2 mM adenosine 5'-(β , γ -imido)triphosphate (AMP-PNP). Where indicated, an ubiquitylation mixture that contained 0.2 μ M recombinant E1 (Enzo Life Sciences) 1 μ M UbcH10, and 100 μ M ubiquitin was added. Following incubation at 23 °C with shaking at 1,400 rpm for the time periods indicated, reaction products were processed as follows. For the estimation of MCC components that remained bound to APC/C, α -Cdc27 beads were washed three times with 1-mL portions of a buffer that contained 50 mM Tris-HCl (pH 7.2), 1 mg/mL BSA, 300 mM NaCl, and 1% Nonidet P-40. Alternatively, or in addition, reaction products released from bead-bound APC/C to supernatants were also analyzed as follows: at the end of incubation a mixture of NaCl and Nonidet P-40 were added to final concentrations of 150 mM and 0.5%, respectively, to minimize nonspecific absorption. After brief centrifugation, supernatants were passed through 5- μ m centrifugal filters (Millipore) to ensure separation from beads. All samples were analyzed by immunoblotting with appropriate antibodies.

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