Role of phosphorylation of Cdc20 in p31^{comet}-stimulated disassembly of the mitotic checkpoint complex

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The mitotic checkpoint system delays anaphase until all chromosomes are correctly attached to the mitotic spindle. When the checkpoint is turned on, it promotes the formation of the mitotic checkpoint complex (MCC), which inhibits the ubiquitin ligase anaphase-promoting complex/cyclosome (APC/C). MCC is composed of the checkpoint proteins BubR1, Bub3, and Mad2 bound to the APC/C activator Cdc20. When the checkpoint is satisfied, MCC is disassembled and APC/C becomes active. Previous studies have shown that the Mad2-binding protein p31^{comet} promotes the dissociation of Cdc20 from BubR1 in MCC in a process that requires ATP. We now show that a part of MCC dissociation is blocked by inhibitors of cyclin-dependent kinases (Cdks) and that purified Cdk1–cyclin B stimulates this process. The mutation of all eight potential Cdk phosphorylation sites of Cdc20 partially prevented its release from BubR1. Furthermore, p31^{comet} stimulated Cdk-catalyzed phosphorylation of Cdc20 in MCC. It is suggested that the binding of p31^{comet} to Mad2 in MCC may trigger a conformational change in Cdc20 that facilitates its phosphorylation by Cdk, and that the latter process may promote its dissociation from BubR1.

cell cycle | spindle checkpoint

The mitotic (or spindle assembly) checkpoint system ensures the accuracy of chromosome segregation by delaying anaphase initiation until all chromosomes are correctly attached to the mitotic spindle by their kinetochores (1–3). This checkpoint system inhibits the action of the anaphase-promoting complex/cyclosome (APC/C), a ubiquitin ligase that targets for degradation inhibitors of mitotic exit, such as securin and cyclin B (4–6). When the checkpoint is activated, APC/C is suppressed by inhibitory factors such as the mitotic checkpoint complex (MCC), composed of the checkpoint proteins BubR1, Bub3, and Mad2 bound to the APC/C activator Cdc20 (7). Another inhibitor, mitotic checkpoint factor 2 (MCF2), has been separated from MCC (8) but its composition is not known. The molecular events involved in the assembly of APC/C inhibitors when the checkpoint is activated, and in their disassembly when the checkpoint is satisfied, remain obscure.

We have been studying the mechanisms of the release of APC/C from mitotic checkpoint inhibition. For this purpose, we used extracts from nocodazole-arrested cells that faithfully reproduced downstream events of the mitotic checkpoint system (9). When such extracts were incubated in the presence of ATP, APC/C was converted to an active form following a lag period. This process was accompanied by the release of inhibitory factors from APC/C and the disassembly of MCC (8, 9). We found that the hydrolysis of ATP at the β–γ position was required for both for the activation of APC/C and for the disassembly of MCC (10); this was different from the previously described requirement of checkpoint inactivation for ubiquitylation (11, 12), because the latter process involves the cleavage of the α–β bond of ATP (13). We have furthermore observed that β–γ-hydrolyzable ATP is needed for the action of p31^{comet} (a Mad2-binding protein known to be involved in checkpoint silencing and mitotic exit) (14–16) to promote MCC dissociation (17). Surprisingly, this process caused the dissociation of Cdc20 from BubR1 in MCC, even though p31^{comet} binds to Mad2 in this complex (17). The mode of action of ATP in MCC dissociation remained unknown. In the present investigation we examined the possibility that the ATP requirement of MCC dissociation reflects the involvement of phosphorylation of some protein(s) involved.

Results

Cyclin-Dependent Kinase Promotes the Disassembly of MCC. We previously found that ATP was required for exit from mitotic checkpoint (10) and observed that ATP cooperated with the Mad2-binding protein p31^{comet} to promote the disassembly of MCC (17). However, the mode of the action of ATP in these processes remained unknown. We therefore examined the possibility that the ATP dependence of MCC disassembly may reflect the involvement of protein phosphorylation. In the experiment shown in Fig. 1*A*, the influence of protein kinase inhibitors on the disassembly of MCC was examined. For this purpose, we used a previously established assay that followed the release of MCC components from BubR1 immunoprecipitates derived from checkpoint extracts (17). In this partially purified system, the joint supplementation of ATP and p31^{comet} caused synergistic release of Cdc20 and Mad2 from BubR1 (17). We found that the addition of staurosporine, an inhibitor of many protein kinases (18), markedly inhibited the release of MCC components in a similar incubation with ATP (Fig. 1*A*, lane 5 vs. 2), or with both ATP and p31^{comet} (lane 7 vs. 4). Similar results were obtained with p27^{Kip1}, a specific inhibitor of cyclin-dependent kinases (Cdks) (19) (Fig. 1*A*, lanes 6 and 8 vs. 2 and 4). However, in both cases, the suppression of MCC disassembly by the protein kinase inhibitor was partial, even though both inhibitors were supplemented at high, maximally effective concentrations. The inhibition of MCC dissociation by p27^{Kip1} indicated that Cdk action was involved in a part of the process promoted by ATP and p31^{comet}.

The above data on the effects of the Cdk inhibitor suggested that a Cdk is presumably associated with BubR1 immunoprecipitates. Extracts of nocodazole-arrested cells (from which BubR1 immunoprecipitates were prepared) contain high levels of cyclin B-activated Cdk, but little if any cyclin E- or cyclin A-stimulated kinase activities, because these cyclins are degraded at earlier stages of the cell cycle. To test whether Cdk1–cyclin B can indeed stimulate MCC disassembly, we tried to remove this protein kinase from anti-BubR1 immunoprecipitates. As described previously, these immunoprecipitates were prepared by washing with buffer containing low salt concentration (17) (*Methods*), to prevent dissociation of material weakly associated with MCC. We found that low-salt wash BubR1 immunoprecipitates indeed contained considerable levels of cyclin B1 and histone H1 kinase, a measure of Cdk activity (Fig. 1*B*, *Left*); most of this could be removed by washing immunoprecipitates with high-salt buffer (Fig. 1*B*, *Right*), and it thus represented material weakly associated with MCC.

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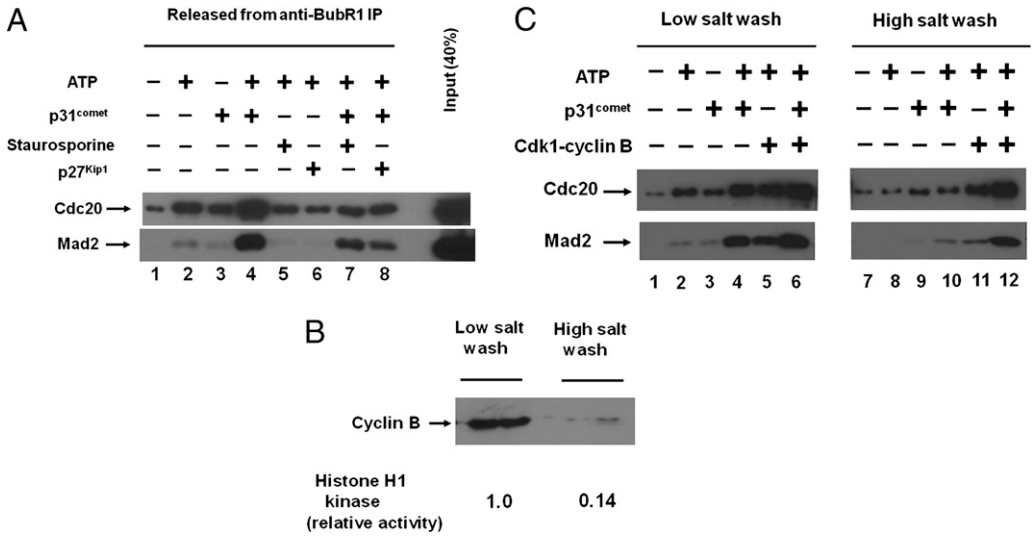

Fig. 1. Influence of protein kinase inhibitors and of Cdk1–cyclin B on the disassembly of MCC. (*A*) Effects of staurosporine and $p27^{Kip1}$ on MCC dissociation. The release of MCC components from anti-BubR1 immunoprecipitates to supernatants was determined as described in *Methods*. Where indicated, additions were at the following concentrations: ATP, 5 mM; $p31^{comet}$, 250 nM; staurosporine, 10 μM; $p27^{Kip1}$, 1.5 μM. (*B*) Influence of high-salt washes of anti-BubR1 immunoprecipitates on levels of associated Cdk1–cyclin B. Anti-BubR1 immunoprecipitates were subjected to washes with buffers containing low- or high-salt concentrations, as described in *Methods*. Samples of 2 μL of beads were subjected to immunoblotting for cyclin B. Histone kinase activities associated with similar immunoprecipitates were determined as described (30) and were expressed relative to that washed with low salt, which was 900 units/μL of beads. (*C*) Cdk1–cyclin B stimulates the release of MCC components from anti-BubR1 immunoprecipitates washed with high-salt buffer. Incubation conditions were as in *A*, except that anti-BubR1 immunoprecipitates were washed with buffers containing low- or high-salt concentrations, as indicated. Where indicated, purified Cdk1-Δ88–cyclin B (1,000 units) was added (30).

Notably, the high-salt wash did not remove significant amounts of MCC components (Mad2 or Cdc20) from BubR1 immunoprecipitates. We then examined the effects of purified Cdk1–cyclin B on MCC dissociation in BubR1 immunoprecipitates subjected to high-salt vs. low-salt wash. As shown in Fig. 1*C*, the addition of the protein kinase stimulated the release of MCC components in both preparations. However, the release of MCC components was more stringently dependent upon the supplementation of Cdk1–cyclin B in the preparation subjected to high-salt wash. Thus, with BubR1 immunoprecipitates incubated with the combination of $p31^{comet}$ and ATP, there was much less release of MCC components in the high-salt wash than in the low-salt wash preparation (Fig. 1*C*, lane 10 vs. 4), and thus the stimulatory effect of Cdk1–cyclin B was much more prominent (lane 12 vs. 6), presumably due to the depletion of endogenous Cdk1–cyclin B by the high-salt wash. The influence of Cdk was most marked in the incubation that contained $p31^{comet}$ (Fig. 1*C*, lane 12), but could also been seen at a lesser degree in its absence (lane 11), which could be due to low amounts of endogenous $p31^{comet}$ bound to MCC, or to a $p31^{comet}$-independent action of Cdk. These results indicate that some phosphorylation reaction catalyzed by Cdk1–cyclin B promotes the dissociation of MCC.

$p31^{comet}$ Stimulates Cdk-Catalyzed Phosphorylation of Cdc20 in MCC. We next questioned the target of the Cdk-catalyzed phosphorylation reaction involved in the dissociation of MCC and whether the phosphorylation is influenced by $p31^{comet}$. For this purpose, BubR1 immunoprecipitate was incubated with [γ-^{32}P]ATP, and the incorporation of ^{32}P-phosphate into proteins was examined. As shown in Fig. 2*A*, lane 1, several proteins in the immunoprecipitate were phosphorylated, including a ~150-kDa protein and a ~55-kDa protein. The 150-kDa protein was possibly BubR1, because this protein kinase is autophosphorylated in immunoprecipitates from mitotic cells (20). The 55-kDa band corresponded to the size of Cdc20. We also found that the addition of $p31^{comet}$ stimulated the phosphorylation of the 55-kDa protein, but not of the 150-kDa protein (Fig. 2*A*, lane 2). In addition, His6-$p31^{comet}$ added to this incubation was also phosphorylated (lane 2, ~34-kDa protein). Because the phosphorylation of Mad2 has been reported to regulate negatively its checkpoint activity (21, 22), we searched for its phosphorylation in the present system, but could not detect phosphorylated protein in the molecular mass region of Mad2 (~25 kDa).

To examine whether the ~55-kDa phosphorylated protein was indeed Cdc20, BubR1 immunoprecipitates labeled with ^{32}P-phosphate from a similar incubation were dissociated with SDS, diluted by buffer containing nonionic detergent, and then reimmunoprecipitated with antibody directed against Cdc20. This reprecipitation procedure identified the 55-kDa phosphorylated protein as Cdc20 (Fig. 2*A*, lanes 3 and 4). The results thus indicated that Cdc20 in MCC is phosphorylated by a protein kinase associated with anti-BubR1 immunoprecipitates, and that this reaction was stimulated by $p31^{comet}$. The protein kinase that phosphorylated Cdc20 was identified as Cdk, by the inhibition of both basal and $p31^{comet}$-stimulated phosphorylation by staurosporine and $p27^{Kip1}$ (Fig. 2*B*). In most experiments, approximately twofold stimulation of Cdc20 phosphorylation by $p31^{comet}$ was observed (Fig. 2*C*).

Phosphorylation of Cdc20 Stimulates Its Release from BubR1. The above results suggested a possible relationship between p31-stimulated phosphorylation of Cdc20 and the disassembly of MCC, but did not prove that this is the mechanism. We attempted to gain direct evidence for this notion by testing the influence of mutations in phosphorylation sites of Cdc20 on its release from BubR1. In the experiment shown in Fig. 3*A*, the validity of this approach was tested with ^{35}S-labeled wild-type Cdc20. In vitro-translated ^{35}S-Cdc20 was exchanged into MCC, which was then isolated by immunoprecipitation with anti-BubR1 antibody (*Methods*). Immunoprecipitates were washed with low-salt buffer and incubated under conditions similar to those in Fig. 1*A*; the release of ^{35}S-Cdc20 to the supernatant was then determined. The dissociation of ^{35}S-Cdc20 from BubR1 was stimulated by ATP (Fig. 1*A*, lane 2) and further stimulated by the combination of ATP and $p31^{comet}$ (lane 4). The release of

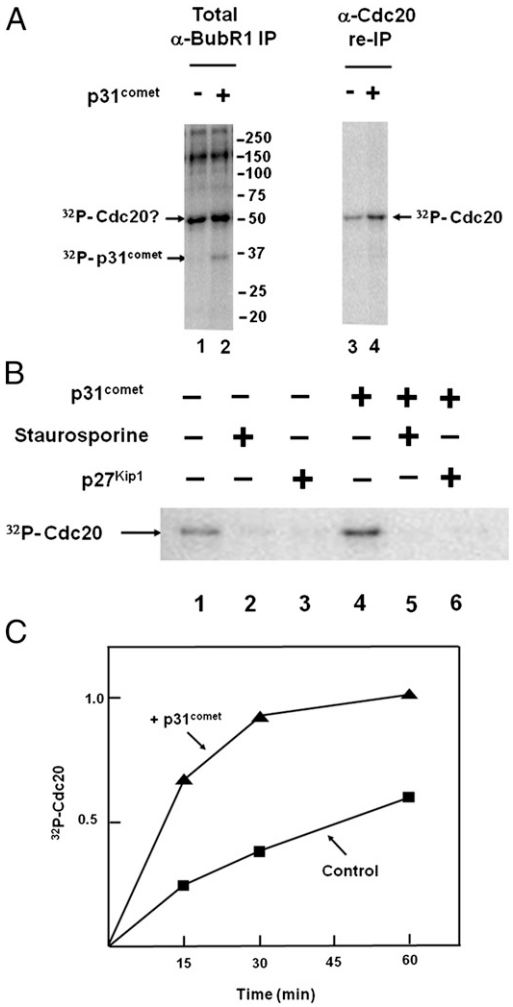

Fig. 2. Influence of p31^{comet} on Cdk-catalyzed phosphorylation of Cdc20 in MCC. (*A*) Incorporation of ^{32}P-phosphate into proteins of anti-BubR1 immunoprecipitates. Immunoprecipitates were incubated with [γ-^{32}P]ATP as described in *Methods*, in the absence or presence of p31^{comet}, as indicated. Samples of the total reaction mixture (lanes 1 and 2) or following reimmunoprecipitation with anti-Cdc20 (lanes 3 and 4; *Methods*) were subjected to SDS/PAGE and storage phosphor autoradiography. The positions of molecular mass marker proteins (kDa) are indicated. (*B*) Effects of protein kinase inhibitors on the phosphorylation of Cdc20 in anti-BubR1 immunoprecipitates incubated with or without p31^{comet}. Experimental conditions were as in *A*. Where indicated, protein kinase inhibitors were added at concentrations similar to those described in Fig. 1*A*. ^{32}P-labeled Cdc20 was isolated by reimmunoprecipitation with anti-Cdc20, as described in *Methods*. (*C*) Effect of p31^{comet} on time course of phosphorylation of Cdc20 in MCC. Experimental conditions were as in *A*, lanes 3 and 4. Samples taken at different times were analyzed for the incorporation of ^{32}P-phosphate into Cdc20. Results are expressed relative to the value obtained with p31^{comet} at 60 min.

labeled Cdc20 was markedly, but not completely, suppressed by the protein kinase inhibitors staurosporine and p27^{Kip1} (Fig. 1*A*, lanes 5–8). These characteristics of the release of recombinant Cdc20 from BubR1 were similar to those of the release of endogenous Cdc20 (Fig. 1*A*).

We next tested the effect of mutation of Cdk phosphorylation sites of Cdc20 on its release from binding to BubR1. Human Cdc20 contains eight Ser/Thr-Pro sequences that are potential Cdk phosphorylation sites. We used a mutant of Cdc20 in which Ser or Thr residues in all eight sites were converted to Ala (Cdc20-8A). Mutant Cdc20 bound to BubR1 was isolated by immunoprecipitation with anti-BubR1 as described previously, and the effect of ATP on its release was then compared with that of wild-type Cdc20 in the presence of p31^{comet}. As shown in Fig. 3*B*, the ATP-stimulated release of Cdc20-8A from BubR1 was much less than that of wild-type Cdc20. As could be expected, the protein kinase inhibitor staurosporine inhibited a great part of the release of wild-type Cdc20, but not of the nonphosphorylatable mutant. The residual release of Cdc20 in the presence of staurosporine was of similar magnitude for the wild-type and mutant proteins. We found that this residual release of Cdc20-8A also required hydrolysis of ATP at the β-γ position, because it did not take place in the presence of the nonhydrolyzable ATP analog adenosine 5′-(β,γ-imido) triphosphate (AMP-PNP; Fig. 3*B*). These findings indicated that a part of the disassembly of MCC required Cdk-catalyzed phosphorylation of Cdc20. Another process involved in MCC disassembly also required ATP hydrolysis, but was not sensitive to the protein kinase inhibitor staurosporine.

Discussion

We were studying the molecular mechanisms of mitotic checkpoint inactivation and observed that ATP is required for the release of APC/C from checkpoint inhibition and for the disassembly of MCC (10). It was furthermore found that ATP cooperates with the Mad2-binding protein p31^{comet} to promote MCC disassembly (17). However, the mode of the action of ATP in these processes remained unknown. In the present investigation, we examined the possibility that ATP may be needed for a phosphorylation process involved in MCC disassembly. We found that the general protein kinase inhibitor staurosporine and the specific Cdk inhibitor p27^{Kip1} inhibit a part of ATP- and p31^{comet}-stimulated release of MCC components from anti-BubR1 immunoprecipitates (Fig. 1*A*). When Cdk1–cyclin B associated with anti-BubR1 immunoprecipitates was depleted by high-salt washes, the supplementation of purified Cdk1–cyclin B strongly stimulated MCC disassembly (Fig. 1*C*). To identify the target of Cdk1-catalyzed phosphorylation, we examined the incorporation of ^{32}P-phosphate into proteins associated with BubR1 immunoprecipitates (Fig. 2*A*). Based on previous reports on the involvement of Mad2 phosphorylation in checkpoint inactivation (21, 22), we searched for the phosphorylation of Mad2, but could not detect it in this system (Fig. 2*A*). However, we noted that the phosphorylation of Cdc20 by Cdk was stimulated by p31^{comet} (Fig. 2). The notion that Cdk-catalyzed phosphorylation of Cdc20 has a role in MCC disassembly was corroborated by the finding that mutation of all Cdk phosphorylation sites of Cdc20 decreased its release from BubR1 to an extent similar to that obtained with staurosporine (Fig. 3*B*).

Based on these observations, we propose the model shown in Fig. 4 for the involvement of p31^{comet} and Cdc20 phosphorylation in MCC disassembly. This scheme also shows further processes in the disassembly to MCC that remain to be elucidated. We assume that p31^{comet} binds to Mad2 in MCC (step 1). This notion is based on the information that p31^{comet} can bind to Cdc20-bound Mad2 (15, 16) and on our previous observation that the binding of p31^{comet} to different MCC-related complexes is proportional to their content of Mad2 (17). We also assume that the binding of p31^{comet} to Mad2 in MCC decreases the affinity of Cdc20 to BubR1, possibly by a conformational change induced in Cdc20. This suggestion is based on the finding that in the absence of ATP, p31^{comet} causes a small but significant release of Cdc20 and Mad2 from BubR1 (17) (Fig. 1*A*). We furthermore assume that the p31^{comet}–Mad2-induced conformational change in Cdc20 exposes some phosphorylation sites in Cdc20 to the action of Cdk (Fig. 4, step 2), which may provide explanation for the stimulatory influence of p31^{comet} on Cdk-catalyzed phosphorylation of Cdc20 in MCC (Fig. 2). It is proposed that this phosphorylation of Cdc20 further decreases its affinity to BubR1, thus accounting for the strong synergistic effect of p31^{comet} and phosphorylation to dissociate MCC (Fig. 4).

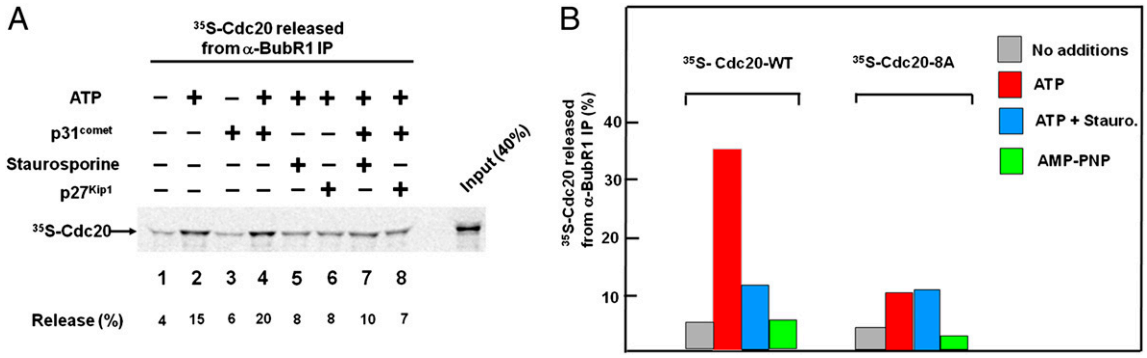

Fig. 3. Phosphorylation of Cdc20 stimulates its release from BubR1. (*A*) Effects of ATP, p31^{comet}, and protein kinase inhibitors on the release of ^{35}S-labeled wild-type Cdc20 from BubR1 immunoprecipitates. ^{35}S-Cdc20 WT was incubated with extracts from nocodazole-arrested cells, and material bound to BubR1 was isolated by immunoprecipitation as described in *Methods*. Anti-BubR1 immunoprecipitates were incubated under conditions similar to those described in Fig. 1*A*, and the release of ^{35}S-Cdc20 was determined by storage phosphor analysis. (*B*) Influence of mutation of Cdk phosphorylation sites of Cdk on its release from BubR1. ^{35}S-labeled Cdc20 WT or Cdc20-8A were incubated with extracts from nocodazole-arrested cells and subjected to immunoprecipitation with anti-BubR1. These immunoprecipitates were incubated with indicated additions at the following concentrations: ATP or AMP-PNP, 5 mM; staurosporine, 10 μM. All incubations contained p31^{comet} (250 nM). The release of ^{35}S-labeled material was expressed as the percentage of that in BubR1 immunoprecipitates at time 0.

Though the proposed sequence of events may account for a pathway for the dissociation of MCC, other pathways apparently also exist and remain to be elucidated. We found that Cdc20 still bound to Mad2 is released in this process (17), so an additional, as yet undefined step presumably separates the components of this subcomplex (Fig. 4, step 3). We also observed that a part of ATP-stimulated release of MCC components was insensitive to staurosporine (Fig. 1*A*), an inhibitor of most protein kinases (18). Similarly, the residual, staurosporine-insensitive release of the nonphosphorylatable mutant Cdc20-8A from BubR1 was promoted by ATP, but not by the nonhydrolyzable analog AMP-PNP (Fig. 3*B*). It thus seems that there is an alternative pathway for MCC dissociation that requires ATP hydrolysis, but is insensitive to the action of staurosporine (Fig. 4, step 4)—this may reflect the involvement of a staurosporine-insensitive protein kinase or of a chaperone-assisted process in MCC dissociation. The existence of a staurosporine-insensitive but ATP-requiring pathway for MCC dissociation may explain the observation that in crude extracts incubated with ATP, staurosporine does not inhibit the dissociation of MCC (10). More investigation is needed to define the mechanisms of this alternate pathway for MCC dissociation.

An additional unsolved problem is that the phosphorylation of Cdc20 is involved not only in MCC disassembly, but also in the opposite process of turning on the mitotic checkpoint, accompanied by the assembly of MCC (23–25). Several protein kinases were proposed to be involved in this process, including Bub1 (23), MAP kinase (24), and Cdk (25). Most sites in Cdc20 phosphorylated by Bub1 are different from Cdk phosphorylation sites, whereas those phosphorylated by MAP kinase are similar. It may be that the phosphorylation of different sites of Cdc20, or a combination of different sites, is involved in MCC disassembly or assembly. We examined the possible influence of mutation of each individual Cdk phosphorylation site on the disassembly of MCC, but none of the eight individual mutations had any significant influence, as opposed to the properties of the 8A mutant. It thus seems that multisite phosphorylation of Cdc20, maybe at a combination of some specific sites, is involved in the disassembly of MCC.

Still another, long-standing mystery is that though p31^{comet} binds to MCC already during active mitotic checkpoint (14, 26, 27), it acts only later to promote checkpoint silencing and exit from mitosis (14, 15, 26–28). It is possible that p31^{comet} is converted to an active form when the checkpoint is turned off, or that an inhibitor of p31^{comet} action is eliminated at that time by some signal linked to the state of the mitotic checkpoint system. Such regulation of p31^{comet} activity would explain what prevents premature activity of this protein to disassemble MCC during active checkpoint, when Cdk activity is high. Much further investigation is needed to gain better understanding of the molecular mechanisms of checkpoint inactivation.

While this paper was under review, the crystal structure of *Schizosaccharomyces pombe* MCC was published (29). The structure shows that Mad2 binds to both Cdc20 and Mad3/BubR1 and thus stabilizes their interaction. p31^{comet} may bind to the same interface and thus may inhibit the assembly of MCC. In this study, truncated derivatives of Cdc20 and BubR1 were used for crystal formation (Cdc20/Slp1 residues 87–488 and Mad3/BubR1 residues 1–223). Therefore, this structure lacks the N-terminal region of Cdc20 that contains four Cdk phosphorylation sites, as well as a great part of the C-terminal region of Mad3/BubR1. Due to this limitation, it is not possible to predict from this structure the Cdk phosphorylation sites that are likely to affect Cdc20–BubR1 interaction. Further structural studies may test our proposal of possible conformational alterations in Cdc20 elicited by p31^{comet} binding and by Cdc20 phosphorylation.

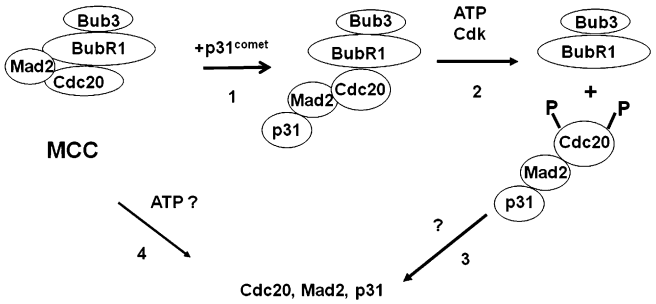

Fig. 4. Proposed sequence of events in the disassembly of MCC (*Discussion*).

Methods

Extracts from nocodazole-arrested HeLa cells were prepared as described previously (9). To isolate MCC free of APC/C, extracts were subjected to sequential immunoprecipitations with anti-Cdc27 followed by anti-BubR1 antibodies [both bound to Affi-Prep Protein A beads (Bio-Rad)], as described (10, 17). These anti-BubR1 immunoprecipitates were used for MCC disassembly experiments. Unless otherwise stated, anti-BubR1 immunoprecipitates were washed 3–4 times with 1-mL portions of a low-salt buffer consisting of 50 mM Tris·HCl (pH 7.2), 1 mg/mL BSA, 20% (vol/vol) glycerol, and 0.5 mM DTT. Where indicated (high-salt wash), immunoprecipitates

were washed twice with a similar buffer that contained 0.3 M NaCl and then twice with low-salt buffer. All immunoprecipitates were finally resuspended in 4 vol of low-salt buffer. The release of MCC components from anti-BubR1 immunoprecipitates to supernatants was determined as described previously (17). Recombinant his6-p31^{comet} (17) and p27^{Kip1} (30) were expressed and purified as described previously. Cdk1-Δ88–cyclin B was prepared and purified as described (31). Cdk activity was determined by the phosphorylation of histone H1 (31).

The phosphorylation of proteins in BubR1 immunoprecipitates was determined with a reaction mixture that contained, in a volume of 30 μL, 25 mm Tris·HCl (pH 7.2), 15 mM $MgCl_2$, 1 mM DTT, 1 mg/mL BSA, 60 mM β-glycerol phosphate, 15 mM *p*-nitrophenyl phosphate, 0.06 mM ATP that contained 2.5 μCi [γ-^{32}P]ATP, and 3 μL anti-BubR1 beads. Following incubation at 23 °C with shaking at 1,400 rpm for 1 h, a 10-μL sample was withdrawn for the estimation of the incorporation of ^{32}P-phosphate into all proteins. Another sample of 15 μL was used for reimmunoprecipitation of Cdc20 as follows. The sample was denatured by boiling in the presence of 1% SDS, diluted 10-fold with PBS containing 1% Nonidet P-40 and 1 mg/mL BSA, and reprecipitated with 5 μg of polyclonal anti-Cdc20 antibody (9) and 10 μL Affi-Prep Protein A beads. Following three washes with PBS that contained 1% Nonidet P-40 and 2 mg/mL BSA, samples were subjected to SDS/PAGE. The incorporation of ^{32}P-phosphate into Cdc20 was detected by storage phosphor analysis.

^{35}S-labeled wild-type Cdc20 and a mutant in which Ser/Thr residues in all eight potential Cdk phosphorylation sites were converted to Ala (Cdc20-8A) were prepared by in vitro transcription-translation with TnT T7 Quick kit (Promega), [^{35}S]methionine (Amersham), and pT7T3 plasmids (32). The plasmid for the expression of Cdc20-8A was prepared from that of Cdc20-7A (32) by introducing the mutation T106A. Because reticulocyte lysates used for in vitro translation contain significant amounts of APC/C, the portion of ^{35}S-Cdc20s bound to APC/C was removed by immunodepletion with anti-Cdc27 antibodies, as described (32).

To estimate the release of ^{35}S-labeled wild-type and mutant Cdc20s from BubR1, they were first exchanged into MCC as follows. Extracts from nocodazole-arrested cell were subjected to immunodepletion with anti-Cdc27 (to prevent binding of labeled Cdc20s to APC/C), and supernatants were mixed with 0.05 vol of in vitro-translated ^{35}S-Cdc20 preparations. The samples were incubated on ice for 3 h and then subjected to immunoprecipitation with anti-BubR1 protein A beads, as described above. The release of ^{35}S-labeled Cdc20 constructs was determined as described for that of endogenous MCC components, and was estimated by storage phosphor analysis.

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