Inhibitory factors associated with anaphase-promoting complex/cylosome in mitotic checkpoint

Ilana Braunstein, Shirly Miniowitz, Yakir Moshe, and Avram Hershko*

Unit of Biochemistry, The Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa 31096, Israel

Contributed by Avram Hershko, January 19, 2007 (sent for review December 20, 2006)

The mitotic (or spindle assembly) checkpoint system ensures accurate chromosome segregation by preventing anaphase initiation until all chromosomes are correctly attached to the mitotic spindle. It affects the activity of the anaphase-promoting complex/cyclosome (APC/C), a ubiquitin ligase that targets inhibitors of anaphase initiation for degradation. The mechanisms by which this system regulates APC/C remain obscure. Some models propose that the system promotes sequestration of the APC/C activator Cdc20 by binding to the checkpoint proteins Mad2 and BubR1. A different model suggests that a mitotic checkpoint complex (MCC) composed of BubR1, Bub3, Cdc20, and Mad2 inhibits APC/C in mitotic checkpoint [Sudakin V, Chan GKT, Yen TJ (2001) *J Cell Biol* 154:925–936]. We examined this problem by using extracts from nocodazole-arrested cells that reproduce some downstream events of the mitotic checkpoint system, such as lag kinetics of the degradation of APC/C substrate. Incubation of extracts with adenosine-5′-(γ-thio)triphosphate (ATP[γS]) stabilized the checkpoint-arrested state, apparently by stable thiophosphorylation of some proteins. By immunoprecipitation of APC/C from stably checkpoint-arrested extracts, followed by elution with increased salt concentration, we isolated inhibitory factors associated with APC/C. A part of the inhibitory material consists of Cdc20 associated with BubR1 and Mad2, and is thus similar to MCC. Contrary to the original MCC hypothesis, we find that MCC disassembles upon exit from the mitotic checkpoint. Thus, the requirement of the mitotic checkpoint system for the binding of Mad2 and BubR1 to Cdc20 may be for the assembly of the inhibitory complex rather than for Cdc20 sequestration.

mitosis | spindle checkpoint | ubiquitin

The mitotic checkpoint (also called spindle assembly checkpoint) system is a surveillance mechanism that delays anaphase initiation until all chromatids are correctly attached to the mitotic spindle via their kinetochores (reviewed in refs. 1–4). Thus, this system ensures accurate segregation of chromosomes in mitosis. When this checkpoint system is turned on, it inhibits the activity of anaphase-promoting complex/cyclosome (APC/C), a multisubunit ubiquitin ligase that is necessary for exit from mitosis. APC/C targets for degradation cell cycle regulatory proteins such as mitotic cyclins and securin, an inhibitor of anaphase initiation (reviewed in refs. 5 and 6). The activity of APC/C is tightly controlled in the cell cycle. It is inactive in the S phase, G_2 phase and in early mitosis and becomes active in late mitosis. The activation of APC/C in mitosis is initiated by its phosphorylation (7). The phosphorylation of APC/C in late mitosis allows its activation by the regulatory protein Cdc20 (8, 9). APC/C^{Cdc20} is the target of the mitotic checkpoint system, so it can become active only after the mitotic checkpoint system has been satisfied. After exit from mitosis, APC/C becomes dephosphorylated, but it is kept active in G_1 by the action of another activator protein, Cdh1 (reviewed in refs. 5 and 6).

The components of the mitotic checkpoint system were initially identified by genetic studies in yeast, and homologous proteins were found in vertebrate cells. These include the checkpoint proteins Mad1, Mad2, and Bub3, protein kinases Bub1, BubR1, and Mps1, as well as kinetochore proteins such as CENP-E. Most checkpoint proteins and protein kinases are concentrated on unoccupied kinetochores when the checkpoint is active, suggesting that some of the important interactions between these proteins may take place at the kinetochores (reviewed in refs. 1–4).

Although these studies, employing approaches of yeast genetics and mammalian cell biology, were very important in describing the properties and identifying many components of this checkpoint system, they could not provide sufficient information on the mode of action of most components and how the system inhibits its downstream target, the APC/C. To gain insight into the molecular mechanisms, biochemical approaches are necessary. Presently available biochemical information is scanty and, in some cases, controversial. It has been shown that Mad2, a protein whose interaction with the APC/C activator Cdc20 is essential for the mitotic checkpoint *in vivo* (10, 11) and inhibits the activity of APC/C^{Cdc20} *in vitro* (12, 13). Mad2 exists in two conformations, of which the closed conformer preferentially binds Cdc20 (14, 15). Elegant models have been proposed according to which the activation of the checkpoint promotes binding of Cdc20 to the closed conformer of Mad2 and thus sequesters Cdc20 from APC/C (refs. 16 and 17; reviewed in ref. 18). However, the concentrations of Mad2 required to inhibit APC^{Cdc20} *in vitro*, including that of the closed conformer (18), are much higher than its physiological levels (19). Another Cdc20-binding checkpoint protein, BubR1, has been reported to inhibit the activity of APC^{Cdc20} *in vitro*, by itself (20) or synergistically with Mad2 (19). In this case, too, the binding of BubR1 to Cdc20 was thought to sequester this protein and thus prevent the activation of APC/C by Cdc20.

As opposed to the above-described "Cdc20 sequestration" models, Sudakin *et al.* (21) proposed that an inhibitory complex, composed of BubR1, Bub3, Cdc20, and Mad2, inhibits the activity of APC/C in mitotic checkpoint. This complex, which was called the mitotic checkpoint complex (MCC), was found to be present and active both in mitosis and in the interphase, but only the mitotic form of APC/C was sensitive to inhibition by MCC. The authors proposed that some modification of APC/C enhances its inhibition by MCC (21). Thus, the MCC model implies that APC/C is inactivated by the mitotic checkpoint, not necessarily because of the sequestration of Cdc20 but because of the action of an inhibitory complex.

Author contributions: I.B. and A.H. designed research; I.B., S.M., Y.M., and A.H. performed research; S.M. and Y.M. contributed new reagents/analytic tools; I.B. analyzed data; and A.H. wrote the paper.

The authors declare no conflict of interest.

Abbreviations: APC/C, anaphase-promoting complex/cyclosome; MCC, mitotic checkpoint complex; ATP[γS], adenosine-5′-(γ-thio)triphosphate; AMP-PNP, adenosine-5′-(β,γ-imido) triphosphate.

*To whom correspondence should be addressed. E-mail: hershko@tx.technion.ac.il.

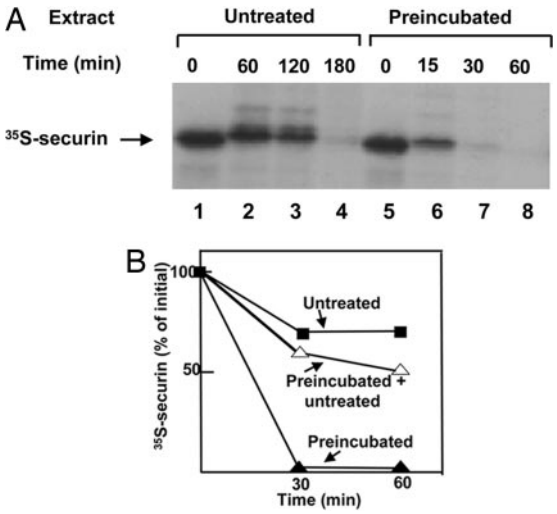

Fig. 1. Existence of labile inhibitor(s) of APC/C in extracts from nocodazole-arrested cells. (*A*) Lag kinetics of the degradation of [^{35}S]securin in extract from nocodazole-arrested cells and influence of preincubation of extract. The degradation of ^{35}S-labeled securin was determined as described in *Methods*, in the presence of either untreated extract (lanes 1–4) or of extract that had been preincubated (23°C, 3 h) with an ATP regeneration mixture (lanes 5–8). (*B*) Inhibition of the degradation of [^{35}S]securin in preincubated extract by untreated extract. The degradation of [^{35}S]securin was determined in the presence of untreated extract (filled squares), preincubated extract (filled triangles), or a 1:1 mixture of untreated and preincubated extracts (open triangles).

In the present investigation, we subjected the mitotic checkpoint system to biochemical analysis, using extracts from nocodazole-arrested cells that faithfully reproduce some of its downstream events. We found that inhibitory factors are specifically associated with APC/C in the checkpoint-arrested state, and identified one of these factors as being similar to MCC. We observed, however, that MCC is disassembled upon exit from the checkpoint-arrested state.

Results

Presence of a Labile Inhibitor of APC/C in Extracts from Nocodazole-Arrested Cells. We first examined whether some characteristics of the mitotic checkpoint system can be reproduced in extracts from nocodazole-arrested mammalian cells. Nocodazole prevents the formation of the mitotic spindle and thus produces a strong mitotic checkpoint signal that inhibits the APC/C. Such soluble extracts from nocodazole-arrested cells do not contain chromosomes (which are removed with the particulate fraction) but still contain some factor(s) that prevent the action of APC/C, as indicated by the kinetics of the degradation of securin, a substrate of APC/C. As shown in Fig. 1*A*, lanes 1–4, when extracts from checkpoint-arrested cells were incubated with [^{35}S]securin at 23°C, there was an initial lag for ≈2 h that was followed by rapid degradation of the labeled substrate. Lag kinetics were observed previously by Sudakin *et al.* (21) in the formation of ubiquitinylated products of cyclin B in similar extracts. The lag resembles the properties of the checkpoint system in intact cells (see *Discussion*). In different preparations of extracts from nocodazole-arrested cells, there was some variability in the duration of the lag period, but the general characteristics of the kinetics of the degradation of [^{35}S]securin remained similar. Preincubation of extracts for 3 h at 23°C before the addition of [^{35}S]securin abolished the initial lag (Fig. 1*A*, lanes 5–8). The rapid degradation of [^{35}S]securin in preincubated extracts was mediated by APC/C, as indicated by the observation that it was effectively competed by unlabeled N-terminal fragment of cyclin B, a good substrate for APC/C (22), but not by a similar fragment that contained mutant destruction box sequence (data not shown). The abolition of the lag in the degradation of [^{35}S]securin in preincubated extracts could be due to the formation of an activator in the course of preincubation or to the decay of a labile inhibitor. To distinguish between these possibilities, we examined the kinetics of the degradation of [^{35}S]securin by a mixture of preincubated and untreated extracts. As shown in Fig. 1*B*, the rapid initial degradation of [^{35}S]securin in preincubated ("activated") extract was effectively prevented by the addition of untreated extract from checkpoint-arrested cells. These findings indicated the presence of labile inhibitor(s) of APC/C-mediated protein degradation in extracts from nocodazole-arrested cells.

Stabilization of Checkpoint-Arrested State by ATP[γS]. Because the biochemical isolation of a labile inhibitor can be greatly facilitated by its prior stabilization, we searched for means to stabilize the checkpoint-arrested state of these extracts. The lability of the inhibitor could be due to several processes, such as the disassembly of an inhibitory complex or the dephosphorylation of factors whose inhibitory influence requires a phosphorylated state. The involvement of protein phosphorylation in the mitotic checkpoint system has been suggested by previous observations such as that several components of the system are protein kinases (1–4) and that the checkpoint-arrested state is associated with the phosphorylation of kinetochore proteins (23). We have first tested the influence of staurosporine, an inhibitor of several protein kinases, on the kinetics of the degradation of [^{35}S]securin in extracts from checkpoint-arrested cells. As shown in Fig. 2*A*, the duration of the lag period in the degradation of [^{35}S]securin was considerably shortened, although not completely abolished, by this protein kinase inhibitor. This suggested that some staurosporine-sensitive protein kinases are involved in the maintenance of the checkpoint-arrested state.

One way to prevent the dephosphorylation of proteins is by the use of the ATP analogue ATP[γS]. This analogue, in which an oxygen in γ-phosphate of ATP is substituted by sulfur, may act with some protein kinases, but thiophosphorylated proteins are not subject to the action of protein phosphatases (24). We therefore tested the influence of preincubation of extracts with ATP[γS] on the kinetics of the degradation of [^{35}S]securin. As shown in Fig. 2*B*, preincubation of extract with ATP[γS] significantly stabilized the inhibited state of APC/C-mediated securin degradation. Thus, between 2 and 3 h of incubation, when [^{35}S]securin was rapidly degraded in untreated extract, it remained stable in extract preincubated with ATP[γS] (Fig. 2*B*). This effect of ATP[γS] was not due to interference with β–γ bond hydrolysis of ATP because preincubation with the nonhydrolyzable β–γ ATP analogue AMP-PNP yielded degradation kinetics essentially similar to those obtained with ATP (Fig. 2*B*). These results are compatible with the interpretation that stable thiophosphorylation of some proteins prevents the activation of APC/C in extracts from checkpoint-arrested cells.

Inhibitory Factor from Checkpoint-Arrested Extract Is Associated with APC/C and Can Be Dissociated with Increased Salt Concentration. We next tried to isolate the putative inhibitor of APC/C from stably checkpoint-arrested extracts. We reasoned that if APC/C is the target of the inhibitor, it is possible that part of the inhibitory factor is directly bound to APC/C. In the experiment shown in Fig. 3*A*, extracts were first incubated with either ATP[γS] ("arrested" extract) or with ATP ("activated" extract) and then were subjected to immunoprecipitation by antibodies directed against Cdc27 (a subunit of APC/C) covalently linked to protein A beads. The activity of immunoprecipitated APC/C to ligate [^{35}S]securin to ubiquitin was assayed in the presence of E1, E2C/UbcH10, and recombinant Cdc20. Without addition of

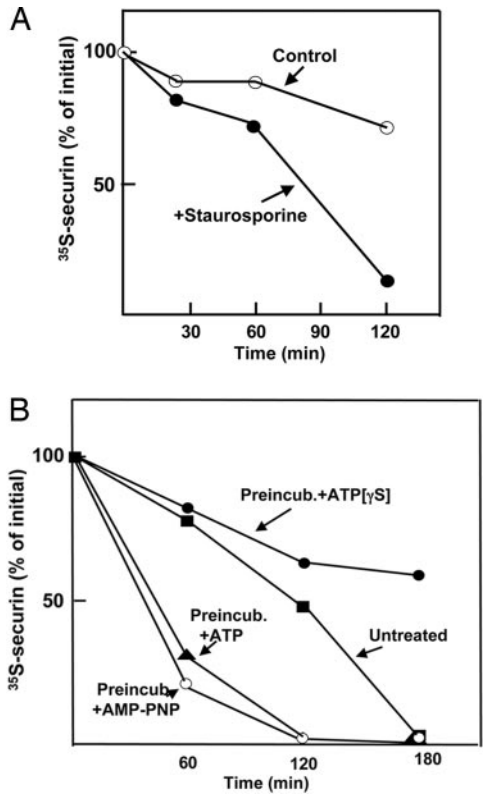

Fig. 2. Protein phosphorylation is required for maintenance of the checkpoint-arrested state of APC/C. (*A*) Influence of staurosporine on the kinetics of the degradation of [^{35}S]securin in extracts from nocodazole-arrested cells. The kinetics of [^{35}S]securin degradation was determined in the presence (filled circles) or absence (open circles) of 10 μM staurosporine. (*B*) Preincubation of extracts with the ATP analogue ATP[γS], but not with AMP-PNP, stabilized the checkpoint-arrested state. Extracts from nocodazole-arrested cells were either untreated or preincubated (23°C, 3 h) with 2 mM ATP, ATP[γS], or AMP-PNP, as indicated, before the supplementation of [^{35}S]securin and ''degradation mixture'' (see *Methods*).

Cdc20, there was very little ubiquitinylation activity in either preparation, even after prolonged incubation (Fig. 3*A*, lanes 2 and 5). This suggested that immunoprecipitated APC/C preparations may not contain sufficient amounts of active Cdc20. With added Cdc20, marked formation of high-molecular-weight [^{35}S]securin–ubiquitin conjugates was observed in immunoprecipitate from activated extract (Fig. 3*A*, lanes 6 and 7). APC/C immunoprecipitated from checkpoint-arrested extract had less, although significant, ubiquitinylation activity under these conditions (Fig. 3*A*, lanes 3 and 4). Quantitative immunoblotting showed that, in both preparations, ≈90% of APC/C was bound to anti-Cdc27 beads. Thus, the results indicated that part of APC/C immunoprecipitated from checkpoint-arrested extracts is in an inhibited state.

The partial suppression of ubiquitinylation activity of APC/C immunoprecipitated from checkpoint-arrested extracts could be due to weak binding to APC/C of inhibitory factor(s). We therefore tried to remove the putative inhibitor by washing the immunoprecipitate bound to anti-Cdc27 beads with a variety of conditions. We found that washing of beads with buffer containing 0.3 M salt caused a significant increase in ubiquitinylation activity of APC/C immunoprecipitated from checkpoint-arrested extracts (Fig. 3*B*, left side). By contrast, a similar salt wash of APC/C immunoprecipitated from activated extract had very little effect on ubiquitinylation activity (Fig. 3*B*, right side). These observations indicated that some inhibitory factor(s) are dissociated from APC/C by increased salt concentration and that this inhibitory material is specific to the checkpoint-arrested state.

We next tried to recover the inhibitory factor from salt eluates of APC/C immunoprecipitates. Anti-Cdc27 immunoprecipitates from arrested and activated extracts were eluted with 0.3 M KCl, eluates were concentrated, and salt was removed by repeated ultrafiltration (see *Methods*). The effects of salt eluates on the activity of APC/C to ubiquitinylate ^{125}I-labeled N-terminal fragment of cyclin B was tested in a purified system consisting of affinity-purified mitotic APC/C (25) and recombinant Cdc20. As shown in Fig. 3*C*, lanes 3 and 4, the addition of increasing amounts of salt eluates from APC/C immunoprecipitated from checkpoint-arrested extracts markedly inhibited the formation of cyclin–ubiquitin conjugates. Similar amounts of salt eluates from anti-Cdc27 immunoprecipitates from activated extracts had much less inhibitory activity (Fig. 3*C*, lanes 5 and 6).

MCC Accounts for Part of APC/C-Associated Inhibitory Activity. We next tried to gain some information on the composition of the inhibitory factor(s) isolated by the approach described above. It has been reported that an MCC consisting of BubR1, Mad2, Cdc20, and Bub3 inhibits the activity of mitotic APC/C (21). Although MCC was found to be present and active in both mitosis and the interphase (21), other investigators reported that MCC components such as Mad2 and BubR1 are preferentially bound to APC/C in mitotic checkpoint (13, 26, 27). We therefore examined whether these proteins are present in salt eluates of anti-Cdc27 immunoprecipitates from checkpoint-arrested and activated extracts. As shown in Fig. 4*A*, lane 1, significant amounts of BubR1, Cdc20, and Mad2 were found in salt eluate of APC/C isolated from checkpoint-arrested extracts. The levels of all these proteins were much less in salt eluate derived from activated extracts by a similar procedure (Fig. 4*A*, lane 2). Thus, the presence of MCC proteins correlated with inhibitory activity in these preparations.

To further examine whether MCC, or an MCC-like complex, indeed account for inhibitory activity, we subjected salt eluate of anti-Cdc27 immunoprecipitate from checkpoint-arrested extracts to immunodepletion by an anti-Cdc20 antibody. As shown in Fig. 4*B*, this treatment removed most Cdc20 from the preparation, as compared with a sham-treated preparation. Immunodepletion of Cdc20 was accompanied by removal of BubR1 and Mad2. These results indicated that BubR1 and Mad2 in this preparation are in a complex with Cdc20.

We next examined the effects of immunodepleted and sham-treated preparations on the inhibition of the ubiquitinylation activity of APC/C^{Cdc20}. As shown in Fig. 4*C*, lanes 3 and 4, increasing amounts of sham-treated salt eluate strongly inhibited the formation of cyclin–ubiquitin conjugates. Similar amounts of anti-Cdc20-treated preparation caused less inhibition (Fig. 4*C*, lanes 5 and 6), indicating that part of inhibitory activity is due to MCC or a related complex. However, it is notable that although immunodepletion with anti-Cdc20 caused nearly complete removal of MCC components (Fig. 4*B*), considerable inhibitory activity remained in this preparation (Fig. 4*C*, lanes 5 and 6). These data suggest that, in addition to MCC, some other inhibitory factors may be associated with APC/C when the mitotic checkpoint system is active.

MCC Is Disassembled in Exit from Checkpoint-Arrested State. We next asked why much less MCC components are associated with APC/C in extracts that had exited from checkpoint arrest (activated extracts) than in checkpoint-arrested extracts (Fig. 4*A*). This could be due to dissociation of MCC from APC/C or to disassembly of MCC itself. To distinguish between these possibilities, we examined the total amount of assembled MCC in differently treated extracts. In the experiment shown in Fig.

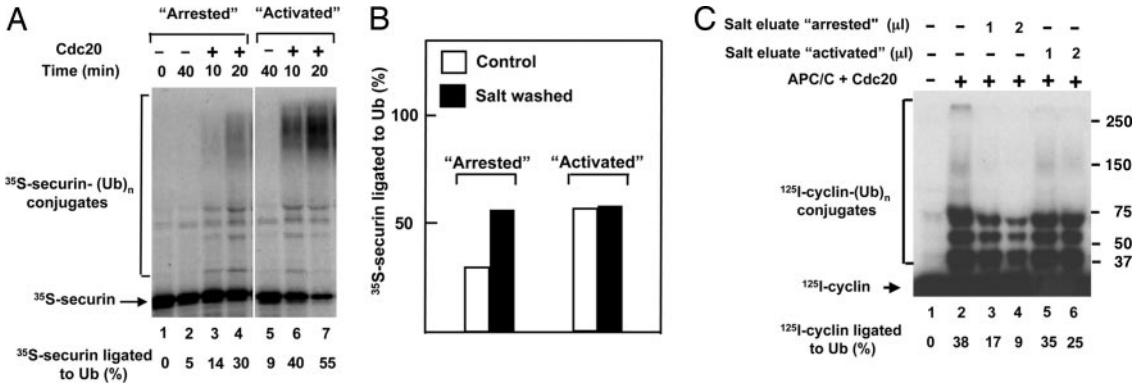

Fig. 3. Inhibitory factor from checkpoint-arrested extract is associated with APC/C and can be dissociated with increased salt concentration. (*A*) Ubiquitinylation of [^{35}S]securin by APC/C immunoprecipitated from checkpoint-arrested and activated extracts. ''Arrested'' and ''activated'' extracts were prepared, and adsorption of APC/C to anti-Cdc27 beads was carried out as described in *Methods*. The ligation of [^{35}S]securin to ubiquitin was carried out as described in *Methods*, with samples of 1-μl anti-Cdc27 beads, for the time periods indicated. Where indicated, 2 ng of recombinant Cdc20 was added. (*B*) Ubiquitinylation activity of APC/C immunoprecipitated from checkpoint-arrested extracts is stimulated by high-salt wash. Adsorption to anti-Cdc27 beads, followed by washes with Buffer A was carried out as described in *Methods*. Subsequently, beads were washed two more times with either Buffer A (open bars), or Buffer A that contained 0.3 M KCl (filled bars). Ubiquitinylation of [^{35}S]securin was estimated in samples of 1 μl of anti-Cdc27 beads incubated for 20 min at 20°C in the presence of 2 ng of Cdc20. (*C*) Salt eluate of APC/C immunoprecipitate from checkpoint-arrested extracts inhibits cyclin–ubiquitin ligation by purified APC/C^{Cdc20}. Salt eluates of anti-Cdc27 immunoprecipitates of checkpoint-arrested and activated extracts were prepared, and the ubiquitinylation of [^{125}I]-cyclin was assayed as described in *Methods*. Numbers on the right indicate the position of molecular mass marker proteins (in kilodaltons).

4*D*, differently treated extracts (untreated, activated by incubation with ATP or arrested by incubation with ATP[γS]) were subjected to immunoprecipitation with anti-Cdc20, and then immunoprecipitates were blotted with antibodies directed against different proteins. Loading controls showed that all immunoprecipitates contained comparable amounts of Cdc20 (Fig. 4*D*, bottom blot). All immunoprecipitates also contained roughly similar amounts of the Cdc27 subunit of APC/C, suggesting that incubation of extracts with ATP or ATP[γS] did not affect appreciably the binding of Cdc20 to APC/C. By contrast, there was a marked decrease in the amounts of BubR1 and Mad2 associated with Cdc20 after incubation of extract with ATP, which had been released from checkpoint arrest (Fig. 4*D*, lane 2). A similar incubation with ATP[γS], which prevents exit from checkpoint, prevented the dissociation of BubR1 and Mad2 from Cdc20 (Fig. 4*D* lane 3). These findings indicated that a large part of MCC disassembles upon exit from the checkpoint-arrested state.

Discussion

In this study, we tried to use an unbiased biochemical approach to gain some insight into the mechanisms by which the mitotic checkpoint system inhibits the activity of the APC/C ubiquitin-protein ligase. Such an approach requires a cell-free system that faithfully reproduces properties of mitotic checkpoint control *in vitro*. It seems that extracts from nocodazole-arrested cells, which do not contain chromosomes, recapitulate at least some downstream events of the mitotic checkpoint system because they carry out the degradation of securin with lag kinetics (Fig. 1*A*). A lag is expected by observations such as that in PtK1cells; there

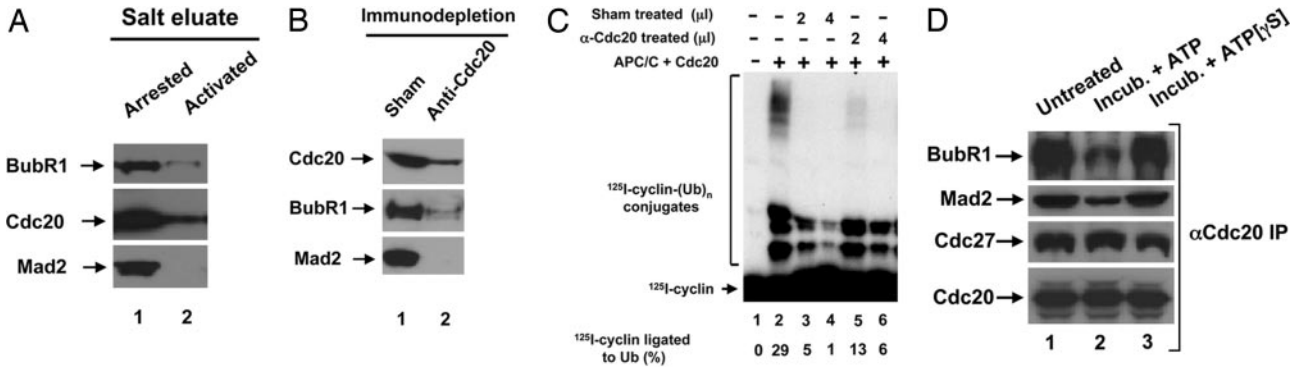

Fig. 4. MCC accounts for a part of APC/C-associated inhibitors. (*A*) Presence of BubR1, Cdc20, and Mad2 in salt eluates of APC/C immunoprecipitates from checkpoint-arrested and activated extracts. Samples of 5 μl of these preparations (see *Methods*) were separated on a 10% polyacrylamide-SDS gel and blotted with the indicated antibodies. (*B*) Immunodepletion by anti-Cdc20 of salt eluate of APC/C immunoprecipitate from checkpoint-arrested extract. Samples of 30 μl of salt eluates were treated with 10 μl of Affi-prep Protein A beads to which 10 μg of affinity-purified polyclonal anti-Cdc20 antibody (provided by Tim Yen) or no antibody (''Sham'') had been previously adsorbed. After rotation at 4°C for 2 h, beads were removed by centrifugation. Samples of 5 μl of supernatants were subjected to immunoblotting as described above. (*C*) Immunodepletion by anti-Cdc20 removes a part of inhibitory activity. The indicated amounts of the preparations described in (*B*) were added to cyclin ubiquitinylation assay in the presence of purified APC/C and Cdc20. (*D*) MCC disassembled in extract incubated with ATP but not with ATP[γS]. Samples of 5 μg of polyclonal anti-Cdc20 antibody (sc-8358; Santa Cruz Biotechnologies) were adsorbed to 10 μl of Affi-prep Protein A beads, and then beads were mixed with 40 μl of either untreated extract from nocodazole-arrested cells (lane 1) or extracts that had been incubated with either ATP (lane 2) or ATP[γS], as described in *Methods*. After rotation at 4°C for 2 h, beads were washed three times with Buffer A and then were suspended in 40 μl of Buffer A that contained 10 mg/ml BSA. Samples of 5 μl of bead suspension were boiled with SDS electrophoresis sample buffer, separated on 10% polyacrylamide-SDS gel, and blotted with the indicated antibodies.

is a delay between the attachment of the last chromatid to the mitotic spindle and the onset of anaphase (reviewed in ref. 28), a process that requires the degradation of securin. This delay is very sensitive to temperature and was prolonged to several hours in incubation of cells at 21–23°C (28), a temperature similar to that used in our *in vitro* incubations. At least part of the lag was due a labile inhibitor, as indicated by the observation that addition of untreated extract to preincubated extract prevented the rapid initial degradation of securin (Fig. 1*C*).

Another property of the cell-free system that is consistent with the characteristics of mitotic checkpoint in intact cells is the involvement of protein phosphorylation in the inhibition of APC/C. Several components of the mitotic checkpoint system are protein kinases (1–4), the phosphorylation of kinetochores has been correlated with the checkpoint-arrested state (23), and phosphorylation of Cdc20 (29–31) and of some checkpoint proteins (1–4) was proposed to be involved in mitotic checkpoint control. Although protein kinase activation is presumably initiated at the kinetochores, and the extracts used in this study do not contain chromosomes, it seems that they still contain some active protein kinases and phosphorylated protein components because the lag is shortened by staurosporine, an inhibitor of some protein kinases (Fig. 2*A*). In addition, the checkpoint-arrested state could be stabilized by ATP[γS], but not by the nonhydrolyzable β–γ ATP analogue AMP-PNP (Fig. 2*B*). This observation is compatible with the interpretation that stable thiophosphorylation of some component(s) prevents exit from the checkpoint-arrested state. Although this assumption remains to be examined, and the putative stably thiophosphorylated components have to be identified, treatment with ATP[γS] was found to be convenient to produce stably checkpoint-arrested extracts.

Our attempt to isolate inhibitors from checkpoint-arrested extracts was based on the assumption that if APC/C is their target, it is possible that part of inhibitory factors is bound to APC/C. We indeed isolated inhibitory factors from immunoprecipitated APC/C by elution with moderately high salt concentration (Fig. 3). The specificity of inhibitory factor(s) in this salt eluate for the checkpoint-arrested state was indicated by the finding that inhibition was much more pronounced with material derived from checkpoint-arrested than from activated extract (Fig. 3*C*). Examination of the composition of this preparation showed that part (although not all) inhibitory activity could be accounted for by a MCC that contains Cdc20, BubR1, and Mad2 (Fig. 4). Inhibitory activity was not simply due to the presence of Mad2 and BubR1 in this preparation because the concentration of Mad2 in MCC is several orders of magnitude lower than that of free Mad2 required to inhibit APC/C (21), whereas the amount of BubR1 in our preparation was ≈30-fold lower than that of recombinant BubR1 required to produce a similar inhibition in the presence of 5 μM Mad2 (data not shown).

Our observations are clearly not compatible with a Cdc20 sequestration model because practically all Mad2 and BubR1 in the inhibitor preparation are already bound to Cdc20 (Fig. 4*B*), and yet this preparation inhibits APC/C activity in the presence of exogenous recombinant Cdc20 (Fig. 4*C*). Our findings are also not in complete agreement with the MCC hypothesis, at least in its original form, because MCC was reported to be present and active in both mitosis and interphase (21), whereas we find that BubR1 and Mad2 dissociate from Cdc20 when extracts are activated by incubation with ATP (Fig. 4*D*). It rather seems that the requirement for binding of Mad2 and BubR1 to Cdc20 when the mitotic checkpoint system is active is not merely for the sequestration of Cdc20 but also to the formation of an MCC complex that inhibits the activity of APC/C^{Cdc20}. Obviously, much more work is needed to determine the composition of the different inhibitory complexes and to delineate the processes that cause the assembly of MCC when the mitotic checkpoint system is turned on and its disassembly upon exit from mitotic checkpoint. The role of various phosphorylation events and the mechanisms by which the inhibitory factors affect the activity of APC/C^{Cdc20} also remain to be elucidated.

Methods

Preparation of Extracts from Nocodazole-Arrested Cells and Treatments with ATPγS or ATP. Extracts from nocodazole-arrested HeLa cells were prepared as described (32), with slight modifications. Briefly, HeLa S3 cells in spinner culture were treated with 2 mM thymidine for 24 h, released from thymidine arrest for 3 h, and then treated with 0.1 μg/ml nocodazole for 11 h. Cells were washed with ice-cold PBS and suspended in 75% of pellet volume of a hypotonic buffer that contained 20 mM Hepes-NaOH (pH 7.6), 5 mM KCl, 1 mM DTT, 10 μg/ml leupeptin, and 10 μg/ml chymostatin. Cells were lysed by freeze–thawing, and lysates were centrifuged at 16,000 × *g* for 1 h. The supernatants were collected, glycerol was added to 10% (vol/vol), and extracts were stored at −70°C in small samples. Protein concentration of extracts was in the range of 15–20 mg/ml.

To prepare stably checkpoint-arrested extracts, the samples were supplemented with 5 mM $MgCl_2$ and 2 mM ATP[γS] (Roche, Indianapolis, IN) and incubated at 23°C for 3 h. Extracts that have exited from checkpoint arrest (activated extracts) were prepared similarly, except that ATP[γS] was replaced by an ATP-regenerating system that contained 10 mM phosphocreatine, 0.1 mg/ml creatine phosphokinase, and 1 mM ATP. In both preparations, some insoluble material appeared after incubation that was removed by centrifugation (16,000 × *g*, 6 min).

Binding of APC/C to Anti-Cdc27 Beads and Elution of APC/C-Associated Material with High Salt. Affinity-purified polyclonal antibodies raised against a C-terminal fragment of the Cdc27 subunit of APC/C were covalently linked to Affi-prep protein A beads (Bio-Rad, Hercules, CA) as described (33). Twenty-microliter samples of anti-Cdc27 beads were washed with Buffer A (50 mM Tris·HCl, pH 7.2/1 mM DTT/10% glycerol/1 mg/ml BSA) and then were mixed with 100 μl of checkpoint-arrested or activated extract. The samples were rotated at 4°C for 1 h, and then beads were washed four times with 1-ml portions of Buffer A.

To elute material associated with APC/C, washed beads were suspended in 1 ml of Buffer A that also contained 0.3 M KCl. Samples were mixed at 4°C for 10 min, and then beads were removed by repeated centrifugation. The supernatants were concentrated by centrifuge ultrafiltration (Centricon-10; Millipore, Billerica, MA), diluted 10-fold with a buffer consisting of 50 mM Tris·HCl (pH 7.2), 1 mM DTT, and 10% glycerol, and concentrated to a volume of ≈50 μl. The salt eluates were stored at −70°C.

Assay of the Degradation of $^{[35}$S]Securin. ^{35}S-labeled securin was produced by *in vitro* transcription–translation as described (34). Reaction mixtures contained 10 μl of extract and 1 μl of 10× degradation mixture that contained 100 mM Tris·HCl (pH 7.6), 50 mM $MgCl_2$, 10 mM DTT, 10 mg/ml ubiquitin, 100 mM phosphocreatine, 1 mg/ml creatine phosphokinase, 5 mM ATP, and 0.1 mg/ml E2C/UbcH10. The reaction was initiated by the addition of 1 μl of [^{35}S]securin, mixtures were incubated at 23°C, and, at various times, samples of 2 μl were withdrawn and rapidly quenched with electrophoresis SDS sample buffer and subjected to SDS-polyacrylamide gel electrophoresis.

Assay of Ubiquitinylation of [^{35}S]Securin or of [^{125}I]Cyclin. The ligation of ubiquitin to [^{35}S]securin was assayed in a reaction mixture that contained in a volume of 10 μl: 40 mM Tris·HCl (pH 7.6), 5 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 1 mg/ml BSA, 2 mM ATP[γS], 5 mg/ml ubiquitin, 1 μM ubiquitin aldehyde, 0.1 μg of E1, 0.2 mg of E2C/UbcH10, 2 ng of recombinant purified Cdc20

(34), APC/C bound to anti-Cdc27 beads as specified, and 0.2 μl [^{35}S]securin. Preparations of [^{35}S]securin had been previously treated with anti-Cdc27 beads to remove APC/C present in reticulocyte lysate (29). After incubation at 20°C with continuous shaking, reaction products were resolved by SDS-polyacrylamide gel electrophoresis. The ubiquitinylation of ^{125}I-labeled N-terminal fragment of cyclin B (33) was assayed in a similar reaction mixture, except that it contained 1 μl of affinity-purified mitotic APC/C (25) with 1 μM okadaic acid, and samples were incubated at 30°C for 30 min.

Immunoblotting and Immunoprecipitation. The following antibodies were used for immunoblotting: Cdc27 (#610455; BD Transduction Laboratories, Lexington, KY); Cdc20, sc-13162 (E7) (Santa Cruz Biotechnologies, Santa Cruz, CA); Mad2 (#610769; BD Transduction Laboratories); and BubR1 (BL1455; Bethyl Laboratories, Montgomery, TX). For immunoprecipitation of Cdc20, we used (as described in figure legends) either polyclonal antibody from Santa Cruz Biotechnologies (sc-8358) or an affinity-purified polyclonal antibody generously provided by Dr. Tim Yen (Fox Chase Cancer Center, Philadelphia, PA).

We thank Drs. Tim Yen and Dvora Ganoth for help and advice. Parts of this work were carried out during the stay of A.H. at the Marine Biological Laboratory (Woods Hole, MA) and at Fox Chase Cancer Center (Philadelphia, PA). This work was supported by the Israel Cancer Research Fund and the Gruss Lipper Foundation.

1. Bharadwaj R, Yu H (2004) *Oncogene* 23:2016–2027.
2. Musacchio A, Hardwick KG (2002) *Nat Rev Mol Cell Biol* 3:731–740.
3. Kops GJP, Weaver BAA, Cleveland DW (2005) *Nat Rev Cancer* 5:773–785.
4. Chan GJ, Liu ST, Yen TJ (2005) *Trends Cell Biol* 11:589–598.
5. Zachariae W, Nasmyth K (1999) *Genes Dev* 13:2039–2058.
6. Peters JM (2006) *Nat Rev Mol Cell Biol* 7:644–656.
7. Lahav-Baratz S, Sudakin V, Ruderman JV, Hershko A (1995) *Proc Natl Acad Sci USA* 92:9303–9307.
8. Shteinberg M, Protopopov Y, Listovsky T, Brandeis M, Hershko A (1999) *Biochem Biophys Res Commun* 260:193–198.
9. Rudner AD, Murray AW (2000) *J Cell Biol* 149:1377–1390.
10. Hwang LH, Lau LF, Smith DL, Mistrot CA, Hardwick KG, Hwang ES, Amon A, Murray AW (1998) *Science* 279:1041–1044.
11. Kim SH, Lin DP, Matsumoto S, Kitazono A, Matsumoto T (1998) *Science* 279:1045–1047.
12. Li Y, Gorbea C, Mahaffey D, Rechsteiner M, Benezra R (1997) *Proc Natl Acad Sci USA* 94:12431–12436.
13. Fang G, Yu H, Kirschner MW (1998) *Genes Dev* 12:1871–1883.
14. Sironi L, Mapelli M, Knapp S, DeAntoni A, Jeang KT, Musacchio A (2002) *EMBO J* 21:2496–2506.
15. Luo X, Tang Z, Rizo J, Yu H (2002) *Mol Cell* 9:59–71.
16. De Antoni A, Pearson CG, Cimini D, Canman JC, Sala V, Nezi L, Mapelli M, Sironi L, Faretta M, Salmon ED, Musacchio A (2005) *Curr Biol* 15:214–225.
17. Luo X, Tang Z, Xia G, Wassman K, Matsumoto T, Rizo J, Yu H (2004) *Nat Struct Mol Biol* 11:338–345.
18. Nasmyth K (2005) *Cell* 120:739–746.
19. Fang G (2002) *Mol Biol Cell* 13:755–766.
20. Tang Z, Bharadwaj R, Li B, Yu H (2001) *Dev Cell* 1:227–237.
21. Sudakin V, Chan GKT, Yen TJ (2001) *J Cell Biol* 154:925–936.
22. Yamano H, Gannon J, Mahbubani H, Hunt T (2004) *Mol Cell* 13: 137–147.
23. Waters JC, Chen RH, Murray AW, Gorbsky, GJ, Salmon ED, Nicklas RB (1999) *Curr Biol* 9:649–652.
24. Eckstein F (1985) *Annu Rev Biochem* 54:367–402.
25. Hershko A (2005) *Methods Enzymol* 398:170–175.
26. Hardwick KG, Johnston RC, Smith DL, Murray AW (2000) *J Cell Biol* 148:871–882.
27. Morrow CJ, Tighe A, Johnson VL, Scott MIF, Ditchfield C, Taylor SS (2005) *J Cell Sci* 118:3639–3652.
28. Rieder CL, Maiato H (2004) *Dev Cell* 7:637–651.
29. Yudkovsky Y, Shteinberg M, Listovsky T, Brandeis M, Hershko A (2000) *Biochem Biophys Res Commun* 271:299–304.
30. Chung E, Chen RH (2003) *Nat Cell Biol* 5:748–753.
31. Tang Z, Shu H, Oncel D, Chen S, Yu H (2004) *Mol Cell* 16:387–397.
32. Rape M, Kirschner MW (2004) *Nature* 432:588–595.
33. Golan A, Yudkovsky Y, Hershko A (2002) *J Biol Chem* 277:15552–15557.
34. Eytan E, Moshe Y, Braunstein I, Hershko A (2006) *Proc Natl Acad Sci USA* 103:2081–2086.