Mad2-Independent Inhibition of APC<sub>Cdc20</sub> by the Mitotic Checkpoint Protein BubR1

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Summary

The mitotic checkpoint blocks the activation of the anaphase-promoting complex (APC) until all sister chromatids have achieved bipolar attachment to the spindle. A checkpoint complex containing BubR1 and Bub3 has been purified from mitotic human cells. Upon checkpoint activation, the BubR1-Bub3 complex interacts with Cdc20. In the absence of Mad2, BubR1 inhibits the activity of APC by blocking the binding of Cdc20 to APC. Surprisingly, the kinase activity of BubR1 is not required for the inhibition of APC<sub>Cdc20</sub>. BubR1 also prevents the activation of APC<sub>Cdc20</sub> in Xenopus egg extracts, and restores the mitotic arrest in Cdc20-overexpressing cells treated with nocodazole. Because BubR1 also interacts with the mitotic motor CENP-E, the ability of BubR1 to inhibit APC may be regulated by kinetochore tension or occupancy.

Introduction

During the cell division cycle, cells first replicate their DNA and then package the DNA into sister chromatids, which are held together by the cohesin protein complex (Nasmyth et al., 2000). After all sister chromatids have achieved bipolar attachment to the mitotic spindle, a ubiquitin ligase called the anaphase-promoting complex or cyclosome (APC) targets the securin protein with poly-ubiquitin chains (King et al., 1996; Zachariae and Nasmyth, 1999). Degradation of the ubiquitinated securin by the proteasome in turn activates the proteolytic activity of the separase (Uhlmann et al., 2000). Proteolytic cleavage of a cohesin protein by the separase destroys the cohesion between the sister chromatids and triggers the onset of anaphase (Uhmann et al., 2000). To ensure the high-fidelity transmission of the genetic material, the timing of sister chromatid separation is closely monitored by the spindle assembly or mitotic checkpoint (Straight and Murray, 1997; Burke, 2000). This checkpoint senses the existence of kinetochores not yet occupied by microtubules (Gorsky and Ricketts, 1993; Li and Nicklas, 1995; Nicklas et al., 1995). A single unattached kinetochore within a cell is sufficient to trigger this checkpoint, resulting in the inhibition of APC, the stabilization of securin, and the delay of the onset of anaphase (Nicklas, 1997).

The molecular mechanism of the mitotic checkpoint has recently begun to unravel. Several molecular components of this pathway were initially identified in budding yeast, including Mad1, Mad2, Mad3, Bub1, Bub2, Bub3, and Mps1 (Hoyt et al., 1991; Li and Murray, 1991; Roberts et al., 1994; Hardwick et al., 1996). Homologs of most of these proteins were then found in other organisms including vertebrates (Chen et al., 1996, 1998; Li and Benezra, 1996; Taylor and McKeon, 1997, Jin et al., 1998; Taylor et al., 1998). Interestingly, the vertebrate homologs of Mad1, Mad2, Bub1, and Bub3 were shown to localize to kinetochores during mitosis (Chen et al., 1996, 1998; Li and Benezra, 1996; Taylor and McKeon, 1997; Taylor et al., 1998; Martinez-Exposito et al., 1999). In addition, a mammalian protein kinase called BubR1 that shares homology with both the yeast Mad3 and Bub1 proteins was also found at the kinetochores in mitosis (Chan et al., 1998, 1999; Jablonski et al., 1998; Taylor et al., 1998). Subsequent genetic and biochemical studies showed that, with the exception of Bub2, all of these molecules are involved in delaying the onset of anaphase in the presence of spindle damage, and may partially account for the proper timing of chromosome segregation during normal mitosis (Taylor and McKeon, 1997; Fraschini et al., 1999; Li, 1999; Bardin et al., 2000; Bloecher et al., 2000; Gardner and Burke, 2000; Pereira et al., 2000). Bub1 and BubR1 are protein kinases and both interact with Bub3 (Taylor et al., 1998). Mad1 is a coiled-coil protein and forms a tight complex with Mad2 throughout the cell cycle (Hardwick and Murray, 1995; Chen et al., 1998, 1999). The biochemical function of Mad2 is relatively well understood. Several lines of evidence have established that Mad2 binds directly to Cdc20, an activator of APC, thereby inhibiting the activity of APC (Li et al., 1997; Fang et al., 1998a, 1998b; Hwang et al., 1998; Kim et al., 1998; Dobles et al., 2000). In contrast, the exact biochemical functions of Bub1, BubR1, Bub3, and Mad1 are not clear. Because these proteins localize to kinetochores in mitosis and because several of them interact physically, it has been postulated that these checkpoint proteins may function as multiprotein complexes (Chan et al., 1999; Brady and Hardwick, 2000; Hardwick et al., 2000).

Elegant experiments on mammalian cells have revealed two extraordinary features of the mitotic checkpoint. First, as a single unattached kinetochore can delay the onset of sister chromatid separation, it must generate an inhibitory signal to block the activity of APC (Rieder et al., 1995). Moreover, this signal needs to be distributed throughout the cell to account for the inhibition of APC that is not associated with the unattached kinetochore (Shah and Cleveland, 2000). Although the nature of this diffusible inhibitory signal has not been established, it is likely to involve the Mad2 protein due to its direct role in the inhibition of APC<sub>Cdc20</sub> and its fast turnover rate at unattached kinetochores (Howell et al., 2000). Second, one of the traits of the unattached kinetochores that the checkpoint senses may be the lack of tension exerted by microtubules (Li and Nicklas, 1995). This notion is further strengthened by the recent finding.
that the kinesin-like motor CENP-E is an essential component of the mitotic checkpoint in mammalian cells and in Xenopus extracts (Abrieu et al., 2000; Yao et al., 2000). CENP-E interacts directly with BubR1 in mitosis, and this interaction may be a part of the force-sensing mechanism (Chan et al., 1999; Yao et al., 2000). However, it is unclear how an imbalance of force can be translated into an activity that inhibits APC.

To gain insight into the roles of the checkpoint proteins in transducing the inhibitory kinetochore signal, we purified a 500 kD BubR1 complex from mitotic HeLa cell lysate using a combination of conventional and immunopurification chromatography. Mass spectrometric analysis revealed that this complex consisted of BubR1 and Bub3 at a 1:1 molar ratio and that Cdc20 was present at a substoichiometric level. This was consistent with the recent finding of Wu et al. that BubR1 associated with Cdc20 in nocodazole-arrested cells (Wu et al., 2000). Purified recombinant BubR1 inhibited the activity of APC in ubiquitination assays at a much lower concentration (Ki = 40 nM) as compared to Mad2 (Ki = 2 μM). BubR1 also blocked the mitotic activation of APC in Xenopus extracts. Surprisingly, the kinase activity of BubR1 was not required for its ability to inhibit APC. Both BubR1 and Mad2 inhibited APC through blocking the binding of Cdc20 to APC. Furthermore, a fragment of BubR1 lacking the Bub3 binding domain blocked the ability of exogenous Cdc20 to prevent mitotic arrest in nocodazole-treated HeLa cells. Taken together, our findings suggest that BubR1 sequesters Cdc20 and inhibits APC in mitosis. Because BubR1 binds to CENP-E directly, the affinity of the BubR1-Cdc20 interaction at the kinetochores may be regulated by microtubule attachment, thus providing a potential link between the molecular sensor of the checkpoint and the inhibition of APC.

**Results**

**Purification of a Mitotic Checkpoint Complex**

Several mitotic checkpoint proteins, including Bub1, BubR1, Bub3, Mad1, Mad2, and Mad3, were reported to form various complexes either in yeast or in mammalian cells (Chan et al., 1999; Brady and Hardwick, 2000; Hardwick et al., 2000). We therefore examined the fractionation profiles of several proteins involved in this pathway on a gel filtration column. Bub1 eluted as part of a 1,000 kD complex in HeLa cells treated with nocodazole, which depolymerized microtubules and activated the spindle assembly checkpoint (Figure 1A). However, the Bub1 complex was also present in cells arrested at the G1/S boundary by thymidine. The fractionation profiles of APC2 and Mad2 in mitotic and G1/S lysates did not vary, either. In contrast, while BubR1 existed as part of a 500 kD complex in G1/S cell lysate, a significant portion of BubR1 was incorporated into a larger complex (1,500 kD) when the checkpoint was activated. Interestingly, the majority of Cdc20 eluted around 250 kD in G1/S lysate. Upon checkpoint activation, Cdc20 formed two additional larger complexes, which appeared to coelute with the two forms of BubR1 complexes in the same lysate (Figures 1A and 1B).

Because BubR1 and Cdc20 exhibited a similar fractionation profile in mitosis, we decided to purify the 500 kD BubR1 complex from nocodazole-treated HeLa cells. Using a combination of conventional and immunopurification chromatography, the BubR1 complex was purified to homogeneity (Figure 1C). Based on Coomassie staining, only two bands, p150 and p40, appeared to be present at stoichiometric levels (Figure 1D). Identical banding patterns were observed for two different antibodies against BubR1. The p150 and p40 proteins were subjected to tryptic digestion followed by liquid chromatography and tandem mass spectrometry (LC/MS/MS) analysis and were identified with high confidence as human BubR1 and human Bub3, respectively. A total of 18 peptides spanning 17.7% of the entire length of BubR1 were identified, while seven peptides covering 21.6% of the Bub3 sequence were detected. Several additional bands were present at substoichiometric levels. One of these bands migrating with a molecular mass of 55 kD belonged to human Cdc20, as mass spectrometric analysis identified a peptide corresponding to residues 84–97 of human Cdc20.

The presence of Cdc20 in the mitotic BubR1-Cdc20 complex was confirmed by immunoblotting (Figure 1E). In nocodazole-treated cells, Cdc20 was detected in the anti-BubR1 immunoprecipitates, and vice versa. About 4-fold less of Cdc20 was present in the BubR1 immunoprecipitates from G1/S cells. The increased association between BubR1 and Cdc20 may be due to the fact that the Cdc20 protein is present at higher levels in mitosis. About 4-fold more of Cdc20 appeared to bind to Mad2 in mitosis, as compared to G1/S. However, Mad2 was not detected in the BubR1 immunoprecipitates (Figure 1E).

**BubR1 Inhibits APC in Reconstituted Ubiquitination Assays and in Xenopus Extracts**

We next tested whether the association of BubR1 with Cdc20 affected the activity of APC in vitro. APC with only basol levels activity was immunopurified from interphase Xenopus egg extracts and was incubated with recombinant human Cdc20 protein purified from Sf9 cells, in the presence of human Bub1, BubR1, Bub3, or oligomeric Mad2 proteins. Purified ubiquitin-activating enzyme (E1), UbcH10, ubiquitin, ATP, and a fragment of human cyclin B1 were also included in the assay. Addition of Cdc20 to interphase APC greatly enhanced its ligase activity toward cyclin B1, whereas Mad2 inhibited the activity of APC (Figure 2A). Interestingly, BubR1 also blocked the activity of APC (Figure 2A). As controls, Bub1 and Bub3 had no effect on APC activity.

We next compared the potency of BubR1 and oligomeric Mad2 to inhibit APC. To our surprise, BubR1 inhibited APC at a much lower concentration (Ki = 40 nM) than Mad2 (Ki = 2 μM), using either cyclin B1 (Figure 2B) or human securin (Figure 2C) as substrates. Based on quantitative immunoblotting, we estimated that the total concentrations of BubR1, Mad2, Cdc20, and APC2 in mitotic HeLa cells were around 90 nM, 120 nM, 100 nM, and 80 nM, respectively (see Supplemental Figure S1 at http://www.developmentalcell.com/cgi/content/full/1/2/227/DC1). Therefore, BubR1 can inhibit APC at the physiological concentration. On the contrary, the concentration of Mad2 in mitotic HeLa cells is well below the Ki of 2 μM, and is thus not sufficient to inhibit APC in vivo without intervention from other...
BubR1 Inhibits APC\textsuperscript{Cdc20} Independently of Mad2

Figure 1. Purification of a Mitotic BubR1 Complex
(A) Lysates of HeLa cells arrested at the G1/S boundary by thymidine or in mitosis by nocodazole were fractionated on a Superose 6 gel filtration column and blotted with various antibodies. The elution volume and the native molecular mass standards are indicated.
(B) The S100 supernatant of the mitotic HeLa cells was fractionated on a Superose 6 gel filtration column. Fractions of 0.25 ml each were collected, instead of the 1 ml fractions shown in (A). Only the fractions containing the higher molecular weight species of BubR1, Cdc20, Mad1, and Mad2 were separated by SDS-PAGE and blotted with antibodies against Cdc20, BubR1, Mad2, and Mad1. The two peaks of Cdc20 coeluted with the two peaks of BubR1. Mad2 fractionated more broadly and peaked differently from BubR1. The fractionation profile of Mad2 more closely resembled that of Mad1.
(C) Purification scheme of the BubR1 complex. The BubR1-containing fractions were identified by immunoblotting.
(D) The BubR1-containing Superose 6 fractions were combined and immunoprecipitated with two different anti-BubR1 antibodies. The immunoprecipitates were analyzed by SDS-PAGE followed by Coomassie staining. The bands belonging to BubR1, Bub3, and Cdc20 are labeled. BubR1 migrates as a doublet. The upper band presumably belongs to the phosphorylated form of BubR1.
(E) Lysates of HeLa cells arrested at the G1/S boundary by thymidine or in mitosis by nocodazole were immunoprecipitated by anti-BubR1, anti-Cdc20, or anti-Mad2, and blotted with the indicated antibodies.

We then examined whether BubR1 and Mad2 can act synergistically to inhibit APC. BubR1 and Mad2 appeared to inhibit APC in an additive manner at several concentrations (data not shown). Because the majority of BubR1 forms a 1:1 complex with Bub3 in vivo, we coexpressed BubR1 and Bub3 in Sf9 cells and purified the BubR1-Bub3 complex to homogeneity (Figure 2D). Binding of Bub3 to BubR1 did not affect the ability of BubR1 to inhibit APC\textsuperscript{Cdc20}, as the BubR1-Bub3 complex inhibited APC at similar concentrations (Figure 2B).

BubR1 also inhibited human APC purified from synchronized HeLa cell lysates (Figure 3A). The addition of Cdc20 activated APC in S, G2, and early mitosis (Fang et al., 1998b). When BubR1 protein was added together with Cdc20, it blocked the ability of Cdc20 to stimulate the activities of APC from S, G2, and early mitosis (Figures 3A and 3B). However, BubR1 did not inhibit the activity of APC in late mitosis or G1.

Addition of a nondegradable form of cyclin B, ∆90-cyclin B, is sufficient to drive the interphase Xenopus egg extracts into mitosis, leading to the activation of cyclin B/cdc2 and APC. We therefore checked whether BubR1 inhibited the activity of APC in Xenopus extracts. When BubR1 was added to the interphase extracts together with ∆90-cyclin B, the degradation of an N-terminal fragment of cyclin B1 was effectively blocked (Figure 3C). BubR1 did not block the activation of cyclin B/cdc2 based on the H1 kinase assay (Figure 3D), indicating that BubR1 prevented the activation of APC at a later step. BubR1 also partially inhibited the degradation of cyclin B1 when added to the mitotic extracts (Figure 3E).
Figure 2. BubR1 Inhibits the Activity of APC<sub>CD20</sub>

(A) I-APC was isolated from interphase Xenopus egg extracts and incubated with recombinant human Cdc20 protein, in the presence of buffer (lane 2), BubR1 (lane 3), Bub1 (lane 4), Bub3 (lane 5), and oligomeric Mad2 (lane 6). The ubiquitination activity of APC was assayed with a Myc-tagged N-terminal fragment of human cyclin B1. The reaction mixtures were separated on SDS-PAGE and blotted with the anti-Myc antibody. The positions of the cyclin B1 substrate and the cyclin B1-ubiquitin conjugates are labeled.

(B) Dose-response experiments of Mad2, BubR1, and the recombinant BubR1-Bub3 complex. Increasing concentrations of Mad2, BubR1, or the BubR1-Bub3 complex were added, together with Cdc20, to interphase APC (I-APC) from Xenopus. The concentration ranges of each of the proteins are indicated.

(C) Same as (B), except that human securin was used as the substrate for APC<sub>CD20</sub> and the oligomeric human Mad2 protein was expressed and purified from Sf9 cells.

(D) The recombinant BubR1-Bub3 complex and the BubR1 protein produced in Sf9 cells were purified by an Ni<sup>2+</sup>-NTA column followed by a Superose 6 gel filtration column. The Superose 6 column fractions containing the BubR1-Bub3 complex (gel 1) and BubR1 (gel 2) were separated on SDS-PAGE followed by Coomassie staining. The bands corresponding to BubR1 and Bub3 are labeled.

However, this effect is less profound than that observed with the interphase extracts, consistent with the fact that the mitotic APC was more resistant to the inhibition of BubR1.

**BubR1 Blocks Binding of Cdc20 to APC**

We then investigated the mechanism by which BubR1 inhibited APC. BubR1 effectively blocked the binding of Cdc20 to the interphase APC (Figure 4A). As shown previously, Cdc20 further stimulated the activity of the mitotic APC. BubR1 inhibited the stimulatory effect of Cdc20 on mitotic APC by preventing Cdc20 binding. However, it did not reduce the activity of the mitotic APC down to the basal level of the interphase APC. This is consistent with our findings that BubR1 did not completely inhibit the degradation of cyclin B in mitotic extracts and that BubR1 failed to block the activity of APC isolated from mitotic HeLa cell lysates. Therefore, BubR1 inhibits the activity of APC by blocking the formation of the active APC<sub>CD20</sub> complex. Once the APC<sub>CD20</sub> complex is formed, it becomes more resistant to the actions of BubR1. Mad2 appeared to inhibit APC through a similar mechanism (Figure 4B). In addition, Cdc20 associated with the mitotic APC more strongly than with the interphase APC because the binding of Cdc20 to mitotic APC was only blocked at higher concentrations of BubR1 or Mad2. This is presumably due to the fact that multiple APC subunits become phosphorylated in mitosis, and phosphorylation may increase the affinity of APC toward Cdc20 (Peters et al., 1996; Fang et al., 1998b; Kotani et al., 1999; Kramer et al., 2000).

**The Kinase Activity of BubR1 Is Not Essential for Inhibition of APC**

BubR1 achieved maximal inhibition of APC<sub>CD20</sub> at roughly a 1:1 molar ratio with Cdc20. Furthermore, BubR1 inhibited APC<sub>CD20</sub> in the presence of AMP-PNP (data not shown). Although AMP-PNP can effectively support the ubiquitination reaction, it cannot be used
Figure 3. BubR1 Inhibits the Activity of Human APC\(^{Cdc20}\) and Blocks the Activation of APC in Xenopus Egg Extracts

(A) HeLa cells were synchronized at the G1/S boundary by a double-thymidine block, released into fresh medium, and harvested at the indicated time points after the removal of thymidine (2, 6, 8, 10, 12, and 14 hr). APC was isolated from the synchronized HeLa cell lysates, incubated with Cdc20 in the presence (+) and absence (−) of BubR1, and assayed for ubiquitination activity.

(B) The same cell lysates used in (A) were blotted with anti-cyclin B1 antibody. The cell cycle status of these lysates is labeled above.

(C) The \(\Delta\)90-cyclin B protein was added to the interphase Xenopus egg extract (I-XT) in the presence or absence of BubR1. After 90 min of the addition of \(\Delta\)90-cyclin B, the Myc-tagged N-terminal fragment (1–102) of human cyclin B1 was added. Samples were taken at the indicated time points and blotted with anti-Myc antibody.

(D) The same extracts, prior to the addition of the N-terminal cyclin B fragment, were assayed for histone H1 kinase activity.

(E) Interphase Xenopus egg extract was incubated with \(\Delta\)90-cyclin B for 90 min. The resulting mitotic extract (M-XT) was assayed for its ability to degrade the N-terminal cyclin B1 fragment in the absence or presence of BubR1.

by kinases to phosphorylate substrates. These results suggested that BubR1 did not function catalytically. To confirm this, we constructed a kinase-inactive mutant of BubR1, K795A, which was likely to disrupt its ability to bind ATP. As expected, the K795A mutation abolished the ability of BubR1 to autophosphorylate, suggesting

Figure 4. BubR1 and Mad2 Block the Binding of Cdc20 to APC

(A) APC was isolated from either interphase (I-APC) or mitotic (M-APC) Xenopus egg extracts. The APC beads were incubated with Cdc20 in the presence of increasing concentrations of BubR1. After washing, these beads were assayed for ubiquitination activity and blotted with anti-Cdc20 antibody.

(B) APC was isolated from either interphase (I-APC) or mitotic (M-APC) Xenopus egg extracts. The APC beads were incubated with Cdc20 in the presence of increasing concentrations of oligomeric Mad2. After washing, these beads were assayed for ubiquitination activity and blotted with anti-Cdc20 antibody.
that BubR1-K795A might not possess kinase activity (data not shown). To identify the regions within BubR1 responsible for the inhibition of APC, a series of BubR1 truncation mutants were prepared (Figures 5A and 5B). The kinase-inactive BubR1-K795A mutant inhibited APC\textsuperscript{STOP} with equal efficiency as the wild-type BubR1 protein (Figure 5C). Therefore, the kinase activity of BubR1 was dispensable for the inhibition of APC\textsuperscript{STOP}. Furthermore, two overlapping fragments of BubR1, BubR1a (residues 526–1050) and BubR1f (residues 1–700), inhibited APC at similar concentrations as the intact BubR1 (Figure 5C). This suggested that the region spanning residues 526–700 might be critical for the inhibitory activity of BubR1. Alternatively, BubR1 might contain multiple Cdc20 binding sites. The latter possibility was more consistent with the fact that the BubR1d fragment containing residues 351–700 had no inhibitory activity toward APC (Figure 5C). Therefore, the APC-inhibitory region of BubR1 cannot be localized to a single small domain. However, a fragment of BubR1 lacking the entire kinase domain (BubR1f) was sufficient to inhibit APC, further supporting the notion that the kinase activity of BubR1 was not required for APC inhibition.

**Binding between BubR1 and Cdc20 Requires the Intact Cdc20**

We next examined the binding between Cdc20 and the BubR1 fragments. To qualitatively compare the affinities of the BubR1-Cdc20 and Mad2-Cdc20 interactions, the binding assay between Mad2 and Cdc20 was also repeated. We had previously shown that Mad2 interacted with a small fragment within the N-terminal region of Cdc20 (Luo et al., 2000). Digestion of in vitro-translated \textsuperscript{35}S-labeled Cdc20 with chymotrypsin resulted in two well-defined fragments of roughly 40 kD and 16 kD in size (Figure 6A). Immunoblotting revealed that the 16 kD fragment contained the N terminus of Cdc20, while the 40 kDa fragment corresponded to its C-terminal WD40 domain (data not shown). Therefore, Cdc20 indeed behaved like a two-domain protein. Two truncation mutants of Cdc20 corresponding to the N- and C-terminal domains were then constructed (Figure 6B). The wild-type oligomeric Mad2 interacted strongly with the N-terminal domain of Cdc20, as did a monomeric form of a Mad2 mutant (with the N-terminal ten residues deleted, \DeltaN10-Mad2). As a control, the Mad2 mutant with its C-terminal ten residues deleted (\DeltaC10-Mad2) did not bind to Cdc20. As expected, none of the Mad2 proteins associated with the C-terminal WD40 domain of Cdc20. Unexpectedly, both the monomeric and oligomeric forms of Mad2 interacted only weakly with the intact Cdc20. The weak interaction between Mad2 and the intact Cdc20 was not due to the inability to form the Mad2-Cdc20 complex posttranslationally, because only minor portions (about 20%) of Mad2 and Cdc20 were able to form a complex even when both proteins were coexpressed in Sf9 cells (data not shown). Similar results were also obtained in a yeast two-hybrid assay (data not shown).
BubR1 Inhibits APC\textsuperscript{Cdc20} Independently of Mad2

Figure 6. Binding of Cdc20 to BubR1 and Mad2

(A) \textsuperscript{35}S-labeled Cdc20 protein was digested with 10 \mu g/ml chymotrypsin (Roche) for 10 min at room temperature and separated on SDS-PAGE followed by autoradiography. The bands belonging to the two domains of Cdc20 are labeled.

(B) Schematic drawing of the boundaries of the Cdc20 constructs corresponding to the N-terminal and C-terminal domains of Cdc20.

(C) The wild-type Mad2 oligomer, the monomeric Mad2 protein with its C-terminal ten residues deleted (\textsuperscript{N}C10-Mad2), and the monomeric Mad2 protein with its N-terminal ten residues deleted (\textsuperscript{N}N10-Mad2) were bound to Ni\textsuperscript{2+}-NTA beads and incubated with the \textsuperscript{35}S-labeled Cdc20, the N-terminal domain of Cdc20 (Cdc20N), or the C-terminal domain of Cdc20 (Cdc20C). After washing, the proteins retained on the beads were analyzed by SDS-PAGE followed by autoradiography.

(D) Various BubR1 mutant proteins were bound to Ni\textsuperscript{2+}-NTA beads and incubated with the \textsuperscript{35}S-labeled Cdc20, the N-terminal domain of Cdc20 (Cdc20N), the C-terminal domain of Cdc20 (Cdc20C), or Bub3. Empty Ni\textsuperscript{2+}-NTA beads were used as the control. After washing, the proteins retained on the beads were analyzed by SDS-PAGE followed by autoradiography.

(E) Various BubR1 mutant proteins were bound to Ni\textsuperscript{2+}-NTA beads and incubated with the \textsuperscript{35}S-labeled Bub3 or Cdc20 in the absence or presence of nonlabeled human Cdc20 protein purified from Sf9 cells. After washing, the proteins retained on the beads were analyzed by SDS-PAGE followed by autoradiography.

not shown). Therefore, Mad2 cannot form a complex efficiently with the intact Cdc20 in the absence of other checkpoint proteins.

In contrast, BubR1 did not bind to either the N- or C-terminal domains of Cdc20. However, it interacted strongly with the intact Cdc20 (Figure 6D). The two BubR1 fragments that were sufficient to inhibit APC, BubR1b and BubR1f, also associated with Cdc20. Interestingly, although the BubR1d fragment did not inhibit APC, it appeared to be sufficient for binding to Cdc20. There are several plausible explanations for this finding. The BubR1d fragment might not interact with Cdc20 as strongly as the intact BubR1 or the other functional BubR1 fragments. The difference in affinity cannot be distinguished by our qualitative binding assays. It is also possible that the BubR1d fragment is not properly folded, which generates a nonnative molecular surface that interacts with Cdc20 nonspecifically. Finally, there might be a distinction between the binding of BubR1 to Cdc20 and the ability of BubR1 to inhibit APC\textsuperscript{Cdc20}. BubR1 might inhibit APC\textsuperscript{Cdc20} only when an extensive interaction between BubR1 and Cdc20 is established, which involves multiple contact sites between large segments of the BubR1 and Cdc20 proteins.

We also mapped the region of BubR1 that was responsible for binding to Bub3. As shown in Figure 6D, BubR1a, BubR1d, and BubR1f interacted with Bub3. Therefore, the Bub3 binding region of BubR1 is likely to reside in residues 351–525. This is consistent with earlier findings that residues 392–433 of BubR1 are required for binding to Bub3 (Taylor et al., 1998). We tested whether the interactions of Bub3 and Cdc20 with BubR1 were mutually exclusive. Though nonlabeled Cdc20 protein produced in Sf9 cells efficiently displaced the binding of \textsuperscript{35}S-labeled Cdc20 to BubR1, it did not block the binding of \textsuperscript{35}S-labeled Bub3 to BubR1 (Figure 6E). Therefore, both Bub3 and Cdc20 can simultaneously bind to BubR1 to form a ternary complex. This is consistent with our
Figure 7. BubR1 Inhibits APC<sub>Cdc20</sub> in HeLa Cells

(A) HeLa cells were transfected with either pCS2-GFP alone or pCS2-GFP together with pCS2-Cdc20, treated with nocodazole, and stained with Hoechst 33342. GFP is shown in green and DNA staining is shown in blue.

(B) HeLa cells were transfected with the indicated plasmids together with pCS2-GFP and treated with nocodazole. The transfected cells are shown in green.

(C) The mitotic index of cells transfected with various plasmids. The results were obtained by counting cells in three separate fields with at least 100 cells each and averaged.

(D) A model for the spindle assembly checkpoint. See Discussion for details.

finding that the BubR1-Bub3 complex inhibited APC<sub>Cdc20</sub> did not restore the mitotic arrest (Figures 7B and 7C). Unexpectedly, overexpression of BubR1<sub>d</sub> efficiently as BubR1 alone reduced the mitotic index of transfected cells upon nocodazole treatment. Because BubR1<sub>d</sub> retained BubR1 Counteracts the Effect of Cdc20

Overexpression in Living Cells

In budding yeast, cells overexpressing Cdc20 do not arrest in mitosis in response to spindle damage, presumably because elevated levels of Cdc20 activate APC and allow cells to bypass the mitotic checkpoint (Hwang et al., 1998; Schott and Hoyt, 1998). Overexpression of Cdc20 in mammalian cells caused a similar phenotype (Figure 7A). HeLa cells were transfected with plasmids encoding Cdc20 and GFP, treated with nocodazole at 300 nM for 24 hr, and stained with Hoechst 33342. Upon nocodazole treatment, about 80% of cells transfected with GFP alone arrested in mitosis, as judged by cell shape and DNA morphology (Figure 7). Cotransfection of GFP and Cdc20 greatly reduced the mitotic index (18%) of transfected cells in the presence of nocodazole. We next checked whether BubR1 could restore the mitotic arrest of the Cdc20-overexpressing cells. When cells were cotransfected with Cdc20 and the BubR1b fragment, about 80% of cells arrested in mitosis in the presence of nocodazole (Figure 7). As a control, cotransfection of Cdc20 and the BubR1d fragment that cannot inhibit APC did not restore the mitotic arrest (Figures 7B and 7C). Unexpectedly, overexpression of BubR1d alone reduced the mitotic index of transfected cells upon nocodazole treatment. Because BubR1d retained the ability to bind Bub3, this fragment might interfere with the function of Bub3 in a dominant-negative fashion. Consistent with the hypothesis, cotransfection of Bub3 with BubR1d restored the mitotic arrest in nocodazole-treated cells (Figures 7B and 7C). Taken together, our data suggest that BubR1 can inhibit APC<sub>Cdc20</sub> in living cells and that Bub3 is important for checkpoint signaling.

Discussion

Tremendous progress has been made in the identification of the molecular components of the spindle assembly checkpoint from various organisms. However, how these proteins interact with each other to generate the checkpoint signal in response to unattached kinetochores remains a mystery (Shah and Cleveland, 2000). The results reported here establish that the checkpoint kinase BubR1 can directly inhibit APC<sub>Cdc20</sub> independently of Mad2. Moreover, BubR1 is a much more potent inhibitor of APC<sub>Cdc20</sub> as compared to Mad2 in vitro. Therefore,
our findings suggest that the BubR1-Bub3 complex may act, in a pathway parallel to Mad2, to inhibit APC<sub>Cdc20</sub> (Figure 7D).

**BubR1 as the Mammalian Homolog of Yeast Mad3**  
On the basis of sequence analysis alone, it is difficult to determine whether BubR1 is the ortholog of yeast Mad3 (Murray and Marks, 2001). The homology between BubR1 and the yeast Mad3 protein is restricted to the N-terminal 200 residues; this region also shares sequence similarity to the yeast Bub1 protein. In addition, the yeast Mad3 protein lacks a C-terminal kinase domain. Despite the ambiguity in sequence alignment and the difference in domain structure between BubR1 and Mad3, our biochemical results support the notion that BubR1 is the functional homolog of yeast Mad3. In yeast, Mad3 interacts with Cdc20 physically, and the Mad3-Cdc20 interaction is critical for checkpoint signaling. This is consistent with the data of Wu et al. and our finding that BubR1 binds directly to Cdc20 (Wu et al., 2000). In addition, BubR1 can inhibit the ability of ectopically expressed Cdc20 to prevent mitotic arrest in the presence of spindle damage. More importantly, the kinase activity of BubR1 is dispensable for the inhibition of APC<sub>Cdc20</sub> in vitro. Though BubR1 contains a Bub1-like kinase domain at its C terminus, there is so far no evidence to indicate that the kinase activity of BubR1 is required for its function in the checkpoint pathway. Therefore, in terms of mechanism of action, BubR1 is more closely related to the yeast Mad3 protein than to the yeast Bub1 protein.

In yeast, the N-terminal region (homology region I) of Mad3 is required for its interaction with Cdc20 (Hardwick et al., 2000). Our data suggest that BubR1 might contain multiple Cdc20 binding regions and that binding of BubR1 to Cdc20 might involve an extensive interface, involving the entire Cdc20 protein and large segments of BubR1. At present, we do not fully understand the reason for this discrepancy. However, neither the assays of Hardwick et al. (yeast two-hybrid and immunoprecipitation) nor our in vitro binding assays are quantitative (Hardwick et al., 2000). Therefore, it is entirely possible that some of the fragments of Mad3 and BubR1 might have lost certain Cdc20 binding elements and bind to Cdc20 with weaker affinity than their full-length counterparts. In fact, based on a yeast two-hybrid assay, the homology region I alone of Mad3 interacted with Cdc20 significantly more weakly than the full-length Mad3 (Hardwick et al., 2000). Consistent with our data, truncation of a small C-terminal region (residues 410–515) of Mad3 significantly weakened the interactions between Mad3 and Cdc20 (Hardwick et al., 2000). Alternatively, this discrepancy might be a consequence of the rather divergent amino acid sequences of the yeast and human proteins. Although the C-terminal WD40 domains of the yeast and human Cdc20 proteins are conserved, there is little sequence homology between the N-terminal domains of these two proteins. Because both the N- and C-terminal domains of human Cdc20 are required for binding to BubR1, it is possible that BubR1 may use multiple binding determinants to interact with human Cdc20. Due to sequence differences in the Cdc20 proteins, some of these binding elements may not be strictly conserved between yeast and human. This notion is further supported by the fact that, despite the existence of homology region I in human Bub1, no significant interaction was observed between Bub1 and Cdc20 in HeLa cells (Figure 1A and data not shown).

**Sequestration of Cdc20 by BubR1**  
Based on the genetic and biochemical studies in yeast and metazoans, it has become increasingly clear that the mitotic checkpoint proteins form a complicated signaling network, instead of a linear pathway (Burke, 2000). In several organisms, numerous complexes of checkpoint proteins, including Mad1-Mad2, Bub1-Bub3, and Mad3/BubR1-Bub3, are detected throughout the cell cycle. Other checkpoint complexes, such as BubR1-Bub3-Cdc20 and Mad2-Cdc20, seem to be enriched in mitotic cells, possibly due to the higher concentrations of Cdc20 in mitosis. Upon checkpoint activation, some of these complexes may interact transiently to produce even larger macromolecular assemblies, and such larger complexes may also be important for the inhibition of APC. Therefore, it is critical to dissect the individual contributions of these interactions to checkpoint signaling.

Both Mad2 and BubR1 can interact with Cdc20 and inhibit APC<sub>Cdc20</sub> in vitro. What are the physiological functions of these interactions? One simple model is that Mad2 and BubR1 act in parallel pathways and sequester different pools of Cdc20 to block the activation of APC. Both proteins are required to effectively inhibit APC<sub>Cdc20</sub> in living cells. Consistent with this sequestration model, both Mad2 and BubR1 are present at relatively high concentrations in cells. The concentrations of Mad2 and BubR1 in HeLa cells are estimated to be 120 nM and 90 nM, respectively, which are comparable to the concentration of Cdc20 (100 nM) in mitotic cells (see Supplementary Figure S1). Furthermore, Mad2 and BubR1 appear to inhibit APC<sub>Cdc20</sub> in an additive fashion in vitro (data not shown). Alternatively, it is possible that, in addition to sequestering Cdc20, binding of BubR1 and Cdc20 helps to recruit Cdc20 to kinetochores at prometaphase, facilitating the formation of other inhibitory checkpoint complexes containing Mad2 and Cdc20. Yet another possibility is that BubR1 and Mad2 might respond to distinct forms of spindle damage during mitosis. As recently suggested by Skoufias et al., Mad2 might sense the attachment of microtubules to kinetochores, whereas BubR1 might respond to the lack of tension at the kinetochores (Skoufias et al., 2001). The hypothesis that BubR1 is involved in tension sensing is consistent with the fact that BubR1 interacts with the mitotic motor CENP-E during mitosis (Abrieu et al., 2000; Yao et al., 2000). It is conceivable that binding of the BubR1-Bub3 complex to Cdc20 might be regulated by CENP-E in response to tension at the kinetochores.

**Experimental Procedures**

**Antibody Production, Immunoprecipitation, and Immunoblotting**  
To generate antibodies against Bub1 and BubR1, several fragments, including Bub1N (residues 1–200), BubR1N1 (residues 1–200), and BubR1N2 (residues 201–400), were expressed as GST fusion proteins in bacteria and purified. The proteins were used to immunize rabbits at Zymed Laboratories. The antisera were purified using the appropriate antigens. The production of anti-APC2, anti-APC3.
Protein Expression in Sf9 Cells and Purification
MS/MS analysis. (Bio-Rad). The protein bands were excised and subjected to LC/HPLC/Xenopus egg extracts, the BubR1 protein at a final concentration of 1 nM. The proteins were further purified by anion exchange or gel filtration twice with XB and assayed for cyclin ubiquitination activity. Each

To purify interphase APC, the anti-APC3 (Cdc27) and anti-Mad2 polyclonal antibodies was reported previously (Fang et al., 1999a, 1999b). A regenerating system, 150 μM of bovine ubiquitin, 5 μM of the Myc-tagged N-terminal fragment of human cyclin B1, 5 μM of human E1, 2 μM of UbcH10, and 2 μM of the APC beads. The reactions were incubated at room temperature for 1 hr, quenched with SDS sample buffer, and analyzed by SDS-PAGE followed by immunoblotting with anti-Myc.

To isolate human APC, HeLa cells were grown in the presence of 2 mM thymidine (Sigma) for 18 hr to arrest the cell cycle at the G1/S boundary, washed with PBS, and grown in fresh medium without thymidine for 8 hr. Cells were then incubated with 2 mM thymidine for another 18 hr, transferred into fresh medium, and harvested at various time points. The cells were lysed with NP-40 lysis buffer. The cell lysates were then incubated with the anti-APC3 antibody beads for 2 hr at 4°C. The beads were then washed, incubated with Cdc20 and other proteins, and assayed for ubiquitination activity as described above.

Acknowledgments
We would like to thank Haizhen Zhu for the production of the GST-Bub1 and GST-BubR1 proteins, Melissa Coldiron for isolating the cDNAs encoding Bub1, BubR1, and Bub3, and Steve Afendis and Steve Madden for mass spectrometry. H.Y. is the Michael L. Rosenzeil Scholar in Biomedical Research. This work is supported in part by the Damon Runyon-Walter Winchell Foundation, the Robert A. Welch Foundation, and the Burroughs Wellcome Fund.

Received January 18, 2001; revised May 21, 2001.

References
Chen, R.H., Brady, D.M., Smith, D., Murray, A.W., and Hardwick,


