The mitotic checkpoint maintains genomic stability by ensuring that chromosomes are accurately segregated during mitosis. When the checkpoint is activated, the mitotic checkpoint complex (MCC), assembled from BUBR1, BUB3, CDC20, and MAD2, directly binds and inhibits the anaphase-promoting complex/cyclosome (APC/C) until all chromosomes are properly attached and aligned. The mechanisms underlying MCC assembly and MCC-APC/C interaction are not well characterized. Here, we show that a novel interaction between BUBR1 and closed MAD2 (C-MAD2) is essential for MCC-mediated inhibition of APC/C. Intriguingly, Arg133 and Gln134 in C-MAD2 are required for BUBR1 interaction. The same residues are also critical for MAD2 dimerization and MAD2 binding to p31comet, a mitotic checkpoint silencing protein. Along with previously characterized BUB1-CDC20 and C-MAD2-CDC20 interactions, our results underscore the integrity of the MCC for its activity and suggest the fundamental importance of the MAD2 αC helix in modulating mitotic checkpoint activation and silencing.

The mitotic checkpoint (or spindle assembly checkpoint) prevents premature sister chromatid segregation by delaying the metaphase-to-anaphase transition in response to defective microtubule attachment at kinetochores. This failsafe mechanism is specified by a group of evolutionarily conserved proteins that includes MAD1, MAD2, BUB1, BUB3, and MPS1 (1). The target of the mitotic checkpoint is anaphase-promoting complex/cyclosome (APC/C), the multi-subunit E3 ubiquitin ligase that promotes anaphase onset through ubiquitylating securin and cyclin B for degradation (2). In APC/C<sub>CDC20</sub>, CDC20 is the activator subunit in the APC/C holoenzyme that also plays major roles in substrate recruitment (3).

Kinetochore lacking proper microtubule attachment or tension emit a diffusible “wait anaphase” signal that has been shown to depend primarily on MAD2 (1). MAD2 can assume two topologically distinct states called open (O) or closed (C) conformers (4, 5). The two conformers exhibit distinct structures at the N and C termini, particularly in the arrangement and position of two C-terminal β-strands and connecting loops. A closed “safety belt”-like loop is present only in C-MAD2 where either MAD1 or CDC20 can bind to form “liganded” C-MAD2. Although the predominant conformer in cells is O-MAD2, during mitosis the MAD1-C-MAD2 complex localized at unattached kinetochores catalytically converts the cytosolic pool of O-MAD2 into C-MAD2 (4, 5). C-MAD2 released from kinetochores can form CDC20-C-MAD2 complexes that may further amplify the production of C-MAD2 by converting additional molecules of O-MAD2 (6).

A MAD2 dimerization domain, mainly involving residues at its αC helix (122–142 residues in human MAD2), is fundamental in mediating O→C-MAD2 conversion. Purified O-MAD2 spontaneously converts into C-MAD2 at a very slow rate (4, 5); therefore recombinant MAD2 contains a mixture of O-MAD2 monomer, O:C heterodimer, and C:C homodimer (7). Acceleration of the O→C conversion has been achieved in vitro with purified liganded C-MAD2, in the form of MAD1-C-MAD2 or CDC20-C-MAD2 complexes. The conversion depends on transient O:C heterodimerization that requires Arg<sup>133</sup> and Gln<sup>134</sup> residues (4–7). Liganded C-MAD2 seems incapable of forming C:C homodimers due to steric clashes at the MAD2 dimerization interface (7). Interestingly, p31<sup>comet</sup>, a negative regulator of the mitotic checkpoint, was shown to exploit the dimerization interface to block O→C-MAD2 conversion during mitosis (8, 9). Through structural mimicry, p31<sup>comet</sup> binds to C-MAD2 at the αC helix thus preventing access of O-MAD2 for heterodimerization (10).

The MCC, composed of BUBR1, BUB3, MAD2, and CDC20, was isolated biochemically from HeLa cells as a factor that can bind and potently inhibit mitotic APC/C (>3000-fold higher activity over recombinant MAD2) (11). The MCC is evolutionarily conserved as homologous complexes have also been identified in <i>Saccharomyces cerevisiae</i>, <i>Schizosaccharomyces pombe</i>, Xenopus laevis, and mouse (12–14). How MCC subunits interact to form the complex and how MCC binds and inhibits APC/C are not fully comprehended (15–17). The current model on MCC composition proposes that MAD2 and the cell cycle-independent BUB1-BUB3 subcomplex do not interact directly, but are brought together with CDC20 as a bridging subunit (18). Once bound to APC/C, MCC efficiently prevents APC/C from binding and ubiquitylating substrates (19). The inhibition of APC/C is thought to arise mainly from the two
Direct Interaction between BUBR1 and C-MAD2

KEN boxes at the N terminus of BUBR1. Although the KEN box is a well characterized degron in many APC/C substrates, BUBR1 may utilize KEN boxes to inhibit APC/C acting as a pseudosubstrate (17, 18, 20, 21). Early in vitro studies showed that high concentrations of both MAD2 and BUBR1 can inhibit the APC/C by binding to and sequestering CDC20 from the APC/C core subunits, and the formation of CDC20-C-MAD2 complex is still accepted as the terminal step of the mitotic checkpoint signal transduction (22–24). It is unclear how MAD2 and BUBR1 coordinate in the MCC to impart the potent APC/C inhibition activity for the whole complex.

EXPERIMENTAL PROCEDURES

**DNA Constructs**—Human full-length BUBR1, MAD2, and CDC20 cDNAs were amplified from a prostate cDNA library (Invitrogen) or freshly prepared reverse transcribed cDNAs provided by Dr. Douglas Leaman (University of Toledo). Full-length cDNAs and fragments were usually cloned into pENTR/D-TOPO first and then subcloned into various destination vectors using the Gateway recombination reactions (Invitrogen). Point mutations were generated using the QuikChange Site-Directed Mutagenesis kit (Stratagene). All constructs were confirmed by DNA sequencing. The features of different protein fragments or mutants are described in supplemental Table S1.

**Cell Culture, Synchronization, and Transfection**—HeLaM, a subline of HeLa, was maintained in DMEM with 10% fetal bovine serum at 37 °C in 5% CO₂. To block cells in prometaphase, HeLaM cells were treated with 2.5 mM thymidine for 24 h and then directly released into medium containing 60 ng/ml nocodazole for 12 h. DNA transfection was performed using polyethylenimine (25) at a DNA-polyethylenimine ratio of 1:3 or FuGENE 6 (Roche).

**Antibodies**—The following antibodies were used at the indicated dilutions: CDC20 (sc-13162, 1:200; Santa Cruz Biotechnology), MAD2 (A300-301A-2, 1:1000; Bethyl), cyclin B1 (sc-245, 1:1000; Santa Cruz Biotechnology), securin (ab3305, 1:100; Abcam), GST (26H1, 1:1000; Cell Signaling), GFP (A11122, 1:500; Santa Cruz Biotechnology). Other antibodies included BUBR1 (mouse monoclonal, 1:1000), BUBR1 (rabbit polyclonal, 1:1000), MAD2 (mouse monoclonal, 1:500), MAD1 (rabbit polyclonal, 1:2000), BUB3 (rabbit polyclonal, 1:1000), CDC27 (rabbit polyclonal, 1:1000), ATM (rabbit polyclonal, 1:200), CDC16 (rabbit polyclonal, 1:1000) and APC7 (rabbit polyclonal, 1:1000) that have been described previously (26, 27). Alkaline phosphate-conjugated goat anti-mouse (A3688) and goat anti-rabbit (A3812) secondary antibodies were purchased from Sigma and used at 1:30,000.

**Cell Lysates, Immunoblotting, Immunoprecipitation, and GST Pulldown**—Cells were lysed in cell lysis buffer (1 × PBS, 10% glycerol, 0.5% Nonidet P-40) supplemented with protease inhibitors (Protease Inhibitor Mixture set III, EDTA-free; Calbiochem) and phosphatase inhibitors (100 mM NaF, 1 mM Na₃VO₄, 60 mM ß-glycerophosphate, 100 mM Microcystin LR). The protein concentration of the lysates was measured using the BCA Protein Assay kit (Pierce). Immunoblotting was used to probe specific proteins in the cell lysates, immunoprecipitates, or in vitro binding assays. In some experiments the blots were scanned, and the intensities of bands of interest were quantified using Kodak Molecular Imaging software. For immunoprecipitation, 200–300 µg of lysates were incubated with appropriate antibodies (0.5–1 µg) at 4 °C for 1 h and then mixed with protein A-agarose beads (RepliGen) for another 1 h. Immune complexes were washed four times with cell lysis buffer containing 250 mM NaCl and then subjected to SDS-PAGE separation. For GST pulldown experiments, cell lysates were added directly to glutathione-agarose (Pierce) and incubated for 1.5 h at 4 °C.

**APC/C Activation Assay Using Concentrated Mitotic Extracts**—The extracts were prepared following Braunstein et al. (28) with minor modifications. Nocodazole-arrested HeLaM cells were harvested by pipetting, washed with ice-cold PBS, and resuspended in 75% of pellet volume of hypotonic buffer (20 mM Hepes-NaOH, pH 7.6, 5 mM KCl, 1 mM DTT) containing protease inhibitors. After repeated freeze-thawing, the cell lysates were centrifuged at 16,000 × g for 1 h. The supernatants were collected, supplemented with glycerol to 10% (v/v), aliquoted, and stored in liquid nitrogen. The protein concentration of the extracts was 15–20 mg/ml. To assay for APC/C activity, 20-µl reaction mixtures contained 10 µl of concentrated mitotic extract, 2 µl of 10X degradation mixture (100 mM Tris-HCl, pH 7.6, 50 mM MgCl₂, 10 mM DTT, 10 mg/ml ubiquitin, 100 mM phosphocreatine, 5 mM ATP, 0.1 mg/ml UbCh10), and 1 µl of 20X creatine phosphokinase (1 mg/ml). Recombinant proteins were added in some experiments. Reactions were incubated at 30 °C, and 3-µl samples were withdrawn at various times, and then rapidly quenched with SDS-PAGE sample buffer. Degradation of cyclin B and securin was followed by immunoblotting.

**Gel Filtration**—Untransfected or 6×HA-MAD2LARQ-transfected mitotic cell lysates were separated on a Superose 6 column (10/300 GL; GE Healthcare). The column was equilibrated and eluted with 20 mM Tris-HCl, pH 7.5, containing 250 mM NaCl and 1 mM DTT. Fractions of 0.5 ml were collected, concentrated by trichloroacetic acid precipitation, and resolved by SDS-PAGE.

**Protein Expression**—GST-tagged BUBR1 (1–371) and CDC20 (111–138), His₆-tagged UbCh10, and wild-type and mutant MAD2 were expressed in *Escherichia coli* BL21-CodonPlus (DE3) Ripl (Stratagene) at 37 °C or 25 °C. His₆-tagged CDC20 and GST-tagged full-length BUBR1 were expressed in Sf9 cells following the manufacturer’s instructions (Invitrogen). Proteins were purified using glutathione-agarose (Pierce) or Probind nickel beads (Invitrogen). The His tag was removed from MAD2 by tobacco etch virus protease cleavage. Concentrations of recombinant proteins were determined by comparing the target band with BSA standards on Coomassie Blue-stained gels.

**In Vitro O→C-MAD2 Conversion**—This was carried out based on Simonetta et al. (6) and illustrated in supplemental Fig. S3. 5 µl of GSH-agarose beads were coated with 25 µl of GST-CDC20(111–138) (0.4 mg/ml) for 2 h at 4 °C. The beads were then washed four times with cell lysis buffer containing 250 mM NaCl. The GST-CDC20(111–138)-coated or mock-treated beads (5 µl) were then incubated with 20 µl of untagged MAD2 WT (~0.1 mg/ml) for 24 h at 25 °C. During the incuba-
tion, the preexistent C-MAD2 in MAD2WT is expected to be captured by GST-CDC20(111–138) and then acts as a catalyst to convert more O-MAD2 into C-MAD2.

**In Vitro Binding Assay**—Recombinant proteins were incubated at 37 °C in 20–H9262 ml reactions in binding buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM MgCl2, 0.5% Nonidet P-40, 5% glycerol, 100 μg/ml BSA) for 1 h. The reactions were then mixed with 5–H9262 ml of glutathione-agarose and gently rocked at 4 °C for 1 h. Beads were washed four times with cell lysis buffer containing 250 mM NaCl before SDS-PAGE.

**Yeast Two-hybrid Assay**—Yeast two-hybrid was carried out as described (29). Coding sequences of two testing proteins were fused with either LexA DNA binding domain or B42 activation domain and used as bait and prey, respectively. The bait and prey DNA constructs were transformed into the yeast strain SKY48. Approximately 1 × 10^6 transformants were plated on a galactose-containing leucine dropout reporter plate. Colony growth, indicating positive protein-protein interactions, was followed for 4 days.

**RESULTS**

**C-MAD2 Delays APC/C Activation in a Chromosome-free Mitotic Cell Extract**—It is widely assumed C-MAD2 amplified by unattached kinetochores constitutes a major “wait anaphase” signal that targets CDC20 to inhibit APC/C activity. To understand the effector formation step of the mitotic checkpoint mechanistically, we used a chromosome-free mitotic extract that partially recapitulates mitotic checkpoint inhibition of the APC/C (11, 28). The extract retained sufficient checkpoint activity to inhibit APC/C for about 15 min before endogenous cyclin B1 and securin were abruptly degraded (Fig. 1A, control). To bypass the signal amplification step that normally requires O-C-MAD2 conversion, the MAD2L13A mutant, which is known to be locked in the C-conformation (10, 30), was purified and added directly into the extract (see supplemental Table S1 for features of protein fragments/mutants used in this study). The addition of MAD2L13A, at 2- and 20-fold excess over endogenous MAD2, extended APC/C inhibition by 15 and 30 min, respectively (Fig. 1, A and B). Importantly, the extended APC/C inhibition correlated with prolonged MCC-APC/C association, as indicated by higher levels of BUBR1 and MAD2 in the CDC27 (an APC/C subunit) immunoprecipitates from 20-fold MAD2L13A supplemented extract, particularly at 30 min (Fig. 1, C and D). In the presence of 20-fold MAD2L13A, cyclin B1 and securin, degradation was observed after 60 min (Fig. 1, A and B), coinciding with >50% of

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**FIGURE 1. C-MAD2 prolongs the lag in APC/C activation in mitotic cell extracts.** A, mitotic extracts prepared from checkpoint-active HeLa cells and incubated in the absence (control) or presence of untagged recombinant MAD2L13A. MAD2L13A was added at 2- and 20-fold of the concentration of endogenous MAD2 in the extract (~23 nm). APC/C activity was monitored by degradation of endogenous cyclin B1 and securin. Samples were taken at the indicated minutes after incubation and analyzed by immunoblotting. ATM and an APC/C subunit CDC27 were loading controls. B, quantitation of cyclin B1 degradation shown in A. Cyclin B1 levels were quantified and normalized against ATM levels in scanned Western blots, and the mean ± S.D. (error bars) from two experiments were plotted versus time. C, mitotic extracts incubated in the absence (control) or presence of 20-fold recombinant MAD2L13A and samples at different times and immunoprecipitated (IP) with anti-CDC27 antibody and probed for MCC and APC/C subunits. D, quantitation of experiment shown in C. BUBR1 and MAD2 levels were normalized against CDC16 (another APC/C subunit) in CDC27 immunoprecipitates and were plotted versus time. The level at the 0 time point in the control experiment was set at 100%. E, kinetics of cyclin B1 degradation in mitotic extracts prepared from untransfected cells (control) or cells transfected with GST-MAD2L13A. GST-MAD2 and endogenous MAD2 were detected simultaneously for comparison of levels. F, untagged recombinant MAD2WT and MAD2C10 added to the mitotic extracts and the rates of cyclin B1 degradation compared.
BUBR1 dissociating from the APC/C (Fig. 1, C and D). Because the MAD2 level in the CDC27 immunoprecipitates was still high at 60 min, the result suggested that simultaneous binding of both BUBR1 and MAD2 is necessary for efficient APC/C inhibition. Delay of APC/C activation was also observed in extracts prepared from GST-MAD2L13A-transfected mitotic cells compared with control extracts (Fig. 1 E).

The delay in activation was specific to C-MAD2 because addition of excess O-MAD2 (MAD2\textsubscript{C10}/H9004C10) did not alter APC/C activation kinetics whereas wild-type (MAD2\textsubscript{WT}), a mixture of O- and C-MAD2, displayed a modest effect (Fig. 1 F).

**C-MAD2 with R133E/Q134A Mutations in the αC Helix Compromises MCC-APC/C Interaction and APC/C Inhibition**—Due to the importance of O:C-MAD2 heterodimerization in the production and amplification of C-MAD2 wait anaphase signals, a dimerization-defective MAD2 (e.g. R133E/Q134A) is unable to support the mitotic checkpoint (31). However, if MAD2 dimerization is only required for O\textsubscript{3}C-MAD2 conversion, addition of a dimerization-defective C-MAD2 (L13A/R133E/Q134A, MAD2\textsubscript{LARQ}) to the chromosome-free mitotic extracts should delay APC/C activation as effectively as MAD2\textsubscript{L13A}. Surprisingly, addition of up to 100-fold molar excess of MAD2\textsubscript{LARQ} did not delay APC/C activation (Fig. 2 A). Similar observations were made when another C-MAD2 mutant (L13Q, MAD2\textsubscript{LQ}) (30) and its dimerization-defective counterpart (L13Q/R133E/Q134A, MAD2\textsubscript{LQRQ}) were tested in the extracts (supplemental Fig. S1 A). The dimerization defect of MAD2\textsubscript{LARQ} was confirmed in the immunoprecipitation experiments shown in Fig. 2 B as it failed to bind endogenous MAD2. Interestingly, the ability of MAD2\textsubscript{LARQ} to bind CDC20

**FIGURE 2.** The R133E/Q134A mutant of C-MAD2 compromises MCC assembly and APC/C inhibition. A, mitotic extracts were incubated in the absence (control) or presence of 100× recombinant MAD2\textsubscript{LARQ} and the kinetics of cyclin B1 and securin degradation compared. B, HeLa cells were transfected with 6×HA-MAD2\textsubscript{L13A}, 6×HA-MAD2\textsubscript{LARQ}, or control and arrested in mitosis with nocodazole. Lysates and anti-HA immunoprecipitates were analyzed for MCC and APC/C subunits by immunoblotting. The arrowhead indicates BUB3, and the asterisk indicates IgG heavy chain. C, HeLa cells were treated as in B, and the mitotic lysates were fractionated through a Superose 6 column. Fractions were probed with indicated antibodies (left). Migration of MCC, APC/C, and MCC-APC/C is indicated with double-headed arrows along with molecular mass standards (vertical arrowheads). The proteins of interest are indicated by horizontal arrowheads if cross-reacting bands can be spotted in the blots. The (8 + 9) ml eluates correspond to the MCC-APC/C complex. D, quantitation of C. The band intensity of each protein (indicated on the left) in each fraction was measured across the elution profiles, and the ratios of proteins in the (8 + 9) ml fractions to the sum intensities of all fractions (8 – 19 ml) were calculated and plotted.
was not compromised \textit{in vivo} (Fig. 2B) or \textit{in vitro} (see Fig. 4A; see also supplemental Fig. S1B for MAD2L13A). Nevertheless, MAD2L13A co-immunoprecipitated only half the levels of BUBR1, BUB3, and CDC27 compared with MAD2L13A (Fig. 2B). Consistently, fractionation of mitotic lysates using size-exclusion chromatography demonstrated that MAD2L13A expression in HeLa cells more effectively promoted MCC-APC/C interaction than MAD2L13A, as evidenced by higher relative enrichment of CDC27, BUBR1, CDC20, HA-tagged and endogenous MAD2 in the fractions corresponding to the MCC/H18528/APC/C supracomplex (8/9 ml eluates, Fig. 2, C and D). Together, these results indicate that Arg133 and Gln134 residues in the αC helix of C-MAD2 are critical for MCC formation and APC/C inhibition in addition to their involvement in O3C-MAD2 conversion.

\textbf{C-MAD2 Interacts Directly with BUBR1—}To delineate further the role of C-MAD2 in MCC assembly, interactions between MCC subunits were examined using purified recombinant proteins at levels comparable with estimated intracellular concentrations ([BUBR1] = 127 μM; [CDC20] = 285 μM; [MAD2] = 230 nM) (24). GST pull-downs were washed and probed alongside inputs with the indicated antibodies. A comparison of full-length BUBR1 and BUBR1(1-371) in binding to MAD2L13A is shown. C, HeLa cells co-transfected with GST-MAD2L13A and either GFP or GFP-BUBR1(1-371) were arrested in mitosis with nocodazole. The lysates and GST pull-downs were probed for CDC20, MAD1, GST-MAD2, GFP, and GFP-BUBR1(1-371). D, yeast two-hybrid assay was performed. The yeast strain SKY48 harboring different combinations of baits and preys were tested for growth on galactose-containing leucine-dropout plates. The \textit{arrowheads} indicate the combinations that produced colonies. E, recombinant MAD2WT was preincubated with GST-CDC20(111-138) immobilized on GSH-agarose beads (+) or beads alone (−) for 24 h. After incubation, GSH-agarose beads were removed by centrifugation, and the resulting supernatants were transferred to new binding reactions containing GST-BUBR1(1-371). GST pull-downs and inputs for the new reactions were probed with the indicated antibodies.
interacted with C-MAD2 but not O-MAD2 in transfected cells (supplemental Fig. S2).

We performed three additional experiments to verify direct BUB1-MAD2 interaction. A MAD2WT mutant was reported to adopt C-conformation preferentially but is incapable of binding CDC20 due to direct involvement of Trp75 in the CDC20-C-MAD2 interaction (7). When expressed in mitotic HeLa cells, the GST-tagged MAD2WT mutant bound to endogenous MAD1, suggesting adoption of C-conformation (Fig. 3C). MAD2WT also indeed failed to interact with endogenous CDC20; however, the mutant associated with co-transfected GFP-BUBR1 (1–371) (Fig. 3C). This in vivo result supports the concept that MAD2 can interact with BUB1 independently of CDC20. Further confirmation of the interaction was conducted using yeast two-hybrid assays (Fig. 3D). When grown on leucine dropout reporter plates, colonies appeared for the following [bait + prey] combinations: [MAD2WT + BUBR1(1–371)], [MAD2L13Q + BUBR1(1–371)], [MAD2L13A + BUBR1(1–371)], [BUBR1(1–371) + MAD2L13A] as well as the positive control [MAD1 + MAD2L13A] (Fig. 3D, arrowheads), whereas vector controls and [MAD2L13A + BUBR1(1–371)] yielded no colonies.

To exclude the possibility that residue substitutions in MAD2L13A and MAD2WT, rather than the conformation of the mutants, enhanced the BUB1-MAD2 interaction, C-MAD2 was generated in an in vitro O→C conversion experiment by preincubating recombinant MAD2WT with immobilized GST-CDC20 (111–138) for 24 h (6) (Fig. 3E; see also supplemental Fig. S3 for explanation of experimental design). Following removal of the GST-CDC20 (111–138) beads, the resulting MAD2-containing supernatant was examined for GST-BUBR1(1–371) binding. GST-BUBR1(1–371) clearly pulled down more MAD2 that had preincubated with CDC20 (111–138) (Fig. 3E, lane 3; compare with lane 2, beads alone), supporting selective binding to C-MAD2.

The R133E/Q134A Mutant Reduces C-MAD2 Binding to BUB1 but Not CDC20—To understand further the functional defects of MAD2LARQ, in vitro binding assays were used to assess the interactions between recombinant MAD2L13A, MAD2LARQ and GST-BUBR1 (1–371) or GST-CDC20 (111–138) following clues from immunoprecipitation results (Fig. 2B). Similar levels of MAD2L13A and MAD2LARQ bound to CDC20 (111–138) following incubation, indicating that both C-MAD2 molecules display similar association with the CDC20 peptide (Fig. 4A, lanes 5 and 6; supplemental Fig. S1B, lanes 5 and 6). The conclusion was also supported with MAD2L13Q and MAD2LARQ mutants (supplemental Fig. S1B, lanes 9 and 10). In contrast, ~5 times more MAD2L13A bound to BUBR1 (1–371) than MAD2LARQ after 1 h of incubation (Fig. 4A, lanes 3 and 4; supplemental Fig. S1B, lanes 3 and 4). Differential bindings to BUBR1 by C-MAD2 with or without additional R133E/Q134A mutations were also observed with full-length BUBR1 (Fig. 4B) and with MAD2LQ and MAD2LARQ mutants (supplemental Fig. S1B, lanes 7 and 8).

**DISCUSSION**

In this study, we presented evidence demonstrating a direct interaction between BUB1 and C-MAD2 and showed that the interaction plays critical roles in MCC-mediated inhibition of APC/C. The results are consistent with the notion that the integr-
The molecular architecture of a functional MCC remains to be characterized further. Two possible explanations are proposed to incorporate our results with existing models (Fig. 4C). In the simplest scheme (model on the left), CDC20 binds MAD2 at the C-terminal safety belt, stabilizing MAD2 in the C-conformation and presenting the αC helix of C-MAD2 for direct interaction with BUBR1. The BUBR1-C-MAD2 interaction, involving residues Arg133 and Gln134 of MAD2 and the N-terminal 371 residues of BUB1, may be facilitated by BUBR1-CDC20 interactions which rely on the KEN boxes in the N-terminal region and/or the second region between residues 400 and 750 of BUB1. Although the two CDC20 binding domains of BUBR1 have been localized to nonoverlapping BUBR1 fragments (17, 18, 22), whether full-length BUBR1 utilizes the two domains to bind two CDC20 molecules or different regions of a single CDC20 molecule remains to be defined. BUB3 forms a cell-cycle independent complex with BUBR1; however, the role of BUB3 in the MCC complex is unclear because the MCC isolated from *S. pombe* lacks detectable BUB3 (13). In the second model (model on the right in Fig. 4C), we speculate that C-MAD2 binds to BUBR1 preferentially as a homodimer, based on the fact that the R133E/Q134A mutant disrupts MAD2 dimerization and meanwhile is unable to interact efficiently with BUBR1 (Figs. 2 and 4 and supplemental Fig. S1). However, homodimerization was suggested to only occur between “unliganded” C-MAD2 (7), it is difficult to imagine the relationship of the C:C-MAD2 homodimer to CDC20-bound C-MAD2 in a fully assembled MCC.

The proposed MCC architecture models open avenues for future study. For example, we realize that binding of either BUBR1 or C-MAD2 to the αC helix of C-MAD2 will block access of O-MAD2, thus formation of the MCC will naturally disassemble and checkpoint silencing (33).

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