MCM-BP regulates unloading of the MCM2–7 helicase in late S phase

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Origins of DNA replication are licensed by recruiting MCM2–7 to assemble the prereplicative complex (pre-RC). How MCM2–7 is inactivated or removed from chromatin at the end of S phase is still unclear. Here, we show that MCM-BP can disassemble the MCM2–7 complex and might function as an unloader of MCM2–7 from chromatin. In *Xenopus* egg extracts, MCM-BP exists in a stable complex with MCM7, but is not associated with the MCM2–7 hexameric complex. MCM-BP accumulates in nuclei in late S phase, well after the loading of MCM2–7 onto chromatin. MCM-BP immunodepletion in *Xenopus* egg extracts inhibits replication-dependent MCM dissociation without affecting pre-RC formation and DNA replication. When excess MCM-BP is incubated with *Xenopus* egg extracts or immunopurified MCM2–7, it binds to MCM proteins and promotes disassembly of the MCM2–7 complex. Recombinant MCM-BP also releases MCM2–7 from isolated late-S-phase chromatin, but this activity is abolished when DNA replication is blocked. MCM-BP silencing in human cells also delays MCM dissociation in late S phase. We propose that MCM-BP plays a key role in the mechanism by which pre-RC is cleared from replicated DNA in vertebrate cells.

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To maintain genome integrity, origins of DNA replication must be activated once and only once per cell cycle. Origins are licensed for replication by the formation of the prereplicative complex (pre-RC). Pre-RC assembly requires loading of the MCM2–7 complex onto DNA through the coordinated action of origin recognition complex (ORC), Cdc6, and Cdt1, and is regulated by Geminin and MCM9 (Remus and Diffley 2009). Once licensing has occurred, MCM2–7 complexes are tightly associated with chromatin, and ORC, Cdc6, and Cdt1 become dispensable to hold MCM2–7 on DNA (Bochman and Schwacha 2009). The MCM2–7 complex serves as a platform to recruit Cdc45 and GINS, thus converting the pre-RC into a pre-initiation complex (pre-IC). Cdc45 and GINS then induce the helicase activity of the MCM2–7 complex, promoting DNA unwinding at origins of DNA replication (for review, see Remus and Diffley 2009).

The MCM2–7 proteins belong to the AAA⁺ superfamily of ATPases and predominantly exist as a heterohexameric complex of ~600 kDa (Forsburg 2004). In addition to such hexameric complexes, MCM subcomplexes (MCM4/6/7, MCM2/4/6/7, and MCM3/5) have also been

reported (Prokhorova and Blow 2000). Although their biological significance is still unclear, MCM4/6/7 is thought to possess core ATPase and DNA helicase activity, whereas MCM2/3/5 provides regulatory roles (Ishimi 1997; Schwacha and Bell 2001). Structural analysis showed that MCM2–7 form a double hexameric ring with head-to-head configuration, and DNA passes through the central channel of the double hexamer (Fletcher et al. 2003; Evrin et al. 2009; Remus et al. 2009). Although it is possible that MCM2–7 oligomerization occurs after its chromatin binding (Maiorano et al. 2000), it is likely that toroid opening is necessary for MCM2–7 unloading from chromatin.

MCM2–7 is displaced from replicated DNA during S phase and cannot be reloaded at origins during S phase, ensuring that each of the many origins can be activated only once during each cell cycle. A number of mechanisms that negatively regulate pre-RC assembly have been identified (Arias and Walter 2007). In metazoans, reloading of MCM2–7 in S phase is prevented by down-regulation of Cdt1 activity in two ways. First, Cdt1 is inactivated by Geminin after activation of the replication origins. Second, Cdt1 is destroyed by ubiquitin-mediated proteolysis in a manner that depends on the initiation of DNA replication. In addition, several inhibitory mechanisms involving Orc1 and Cdc6 have been reported (Delmolino et al. 2001; Mendez et al. 2002). However,

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ORC, Cdc6, and Cdt1 are dispensable for retaining MCM2–7 on chromatin. It is therefore likely that there are additional mechanisms to inactivate the MCM2–7 helicase following S-phase progression.

MCM-BP was identified as a novel MCM-binding protein by tandem affinity purification of MCM proteins from human cells (Sakwe et al. 2007). It is highly conserved among higher eukaryotes. Human MCM-BP can associate with MCM3–7 but not MCM2, and hexameric MCM2–7 complexes lack MCM-BP. A fraction of human MCM-BP binds to chromatin in a cell cycle-dependent manner like other MCM proteins, and silencing of *MCM-BP* by siRNA reduced the amount of MCM4 on chromatin, but cell growth was not significantly impaired (Sakwe et al. 2007). Finally, a mutant of plant MCM-BP has been reported to display G2 cell cycle arrest (Takahashi et al. 2008, 2010). Thus, MCM-BP may have an important role in S-phase progression, but its function, like its effect on the MCM complex, remains unclear.

Here, we characterized the function of MCM-BP using in vitro systems derived from *Xenopus* egg extracts. We find that MCM-BP can disassemble the MCM2–7 hexameric complex and functions as an unloader of MCM2–7 from chromatin after DNA replication. We also show that MCM-BP has a similar function in human cells. Our results show that MCM2–7 hexamer rings can be opened by MCM-BP to promote MCM2–7 dissociation at the end of the replicon synthesis.

Results

Xenopus MCM-BP binds to MCM7 but is not part of the MCM2–7 complex

To characterize the functional properties of MCM-BP in *Xenopus* egg extracts, we prepared and purified rabbit polyclonal antibodies against His¹⁰-tagged, full-length recombinant *Xenopus* MCM-BP, a 626-amino-acid-long protein that is 72% identical to human MCM-BP (Supplemental Fig. S1). This antibody, but not the preimmune serum, specifically recognized a protein with an apparent molecular weight of 70 kDa in *Xenopus* egg extracts (Fig. 1A) and also in vitro translated *Xenopus* MCM-BP (Supplemental Fig. S2A). By comparing the signal in egg extracts with given amounts of recombinant MCM-BP run in parallel, we estimated the concentration of MCM-BP in *Xenopus* egg extracts to be 10 ng/μL or 140 nM (data not shown). In *Xenopus* oocytes, which were arrested at the prophase stage of the first meiotic division, MCM-BP was localized in the nucleus (GV), like MCM proteins (Supplemental Fig. S2B).

To confirm that *Xenopus* MCM-BP is a MCM-binding protein, and to determine its relationship with the MCM2–7 complex, *Xenopus* interphase egg extracts were immunoprecipitated with polyclonal anti-MCM-BP and anti-MCM7 antibodies. The anti-MCM7 antibody coimmunoprecipitated other MCM subunits, as expected, as well as a large amount of MCM-BP (Fig. 1B; Supplemental Fig. S2C). The anti-MCM-BP antibody coprecipitated MCM7 as well as MCM3 and MCM5, but not MCM2,

Figure 1. *Xenopus* MCM-BP binds to MCM proteins. (A) Western blot analysis of 0.5 μL of *Xenopus* interphase egg extracts with the rabbit polyclonal anti-MCM-BP antibody (I) or preimmune serum (PI). (B) MCM-BP associates with MCM proteins in *Xenopus* interphase egg extracts. Immunoprecipitations carried out using anti-MCM-BP (lane 3) or anti-MCM7 (lane 4) antibodies were analyzed by Western blotting using the indicated antibodies. Also shown is a mock immunoprecipitation to determine background signals (lane 2) and the proteins present in untreated egg extracts (0.5 μL) (lane 1). (C) MCM-BP and MCM3 immunoprecipitates from *Xenopus* interphase egg extracts were analyzed by MS. MS profiles were identified using the Mascot search engine. Score: Mascot scores.

and very little MCM4 (Fig. 1B). These data are in agreement with observations in human cells (Sakwe et al. 2007). Mass spectrometry (MS) analysis of the MCM-BP and MCM3 immunoprecipitates confirmed these results (Fig. 1C). Altogether, these data suggest that MCM-BP associates with MCM proteins, but it is not a cofactor of the whole MCM2–7 complex.

To further investigate the composition of the different MCM subcomplexes, *Xenopus* interphase egg extracts were fractionated by sucrose gradient sedimentation. The MCM2–7 complex sedimented as a major 600-kDa complex, and a second complex of ~200 kDa that contained mainly MCM7 was also observed (Fig. 2A). The abundance of this second complex varied with the extracts, as observed previously (Coue et al. 1998; Prokhorova and Blow 2000). Interestingly, this second complex was more abundant in extracts in which DNA replication was finished than in extracts treated with Geminin (Supplemental Fig. S3A), suggesting that the formation of this subcomplex is actively regulated during DNA replication. MCM-BP was not present in the 600-kDa MCM2–7 complex that contains the full MCM2–7 hexamer (Fig. 2A). However, MCM-BP did not sediment at a position corresponding to its

Figure 2. MCM-BP forms subcomplexes with MCMs, mainly with MCM7. (A) Western blot analysis of *Xenopus* interphase egg extracts following fractionation on a linear 7%–19% sucrose gradient (see the Materials and Methods). (B) Low-molecular-weight fractions from the sucrose gradient shown in A (fractions 22–24) were immunoprecipitated using anti-MCM-BP (lane 2) and anti-MCM7 (lane 3) antibodies as indicated. Immunoprecipitated proteins were analyzed by Western blotting using antibodies against MCM7 and MCM-BP. Proteins present in the pool were also analyzed. (C) MCM7 was radiolabeled with ^{35}S -methionine during in vitro translation in a reticulocyte lysate system and then incubated with GST control (lane 1) or GST-MCM-BP (lane 2). GST complexes were isolated with GSH-Sepharose beads and analyzed by autoradiography. Two percent of the reaction was analyzed as input. (D) MCM-BP binds to the conserved MCM box in MCM7. (Left) Schematic representation of MCM7 deletion mutants. GST (lanes 1–6) or GST-MCM-BP (lanes 7–12) was bound to GSH-Sepharose beads and incubated with either Myc-tagged recombinant full-length MCM7 or the various deletion mutants. (Right) Coprecipitated proteins were analyzed by Western blotting using the anti-Myc antibody.

molecular weight (70 kDa), but was detected as a 150- to 200-kDa protein complex, in agreement with the presence of a MCM-BP–MCM7 complex (70 kDa + 81 kDa). Immunoprecipitation of pooled fractions that corresponded to the MCM-BP peak confirmed that MCM-BP was associated with MCM7 (Fig. 2B). MCM3 or MCM4 was hardly coimmunoprecipitated with MCM-BP after the sedimentation (data not shown), probably due to the dissociation of the complex during the long centrifugation (22 h). Indeed, the MCM-BP–MCM7 complex was rather stable, as it was resistant to high-salt elution (0.8 M NaCl) (Supplemental Fig. S3B), whereas MCM2 and MCM4 were mostly eluted in the high-salt fraction (Supplemental Fig. S3B; data not shown). The MCM7 (or MCM4)–MCM-BP complex could be detected in both interphase and mitotic extracts, suggesting that its formation is not cell cycle-dependent (Supplemental Fig. S3C).

The MCM-BP–MCM7 interaction was confirmed by GST pull-down experiments, showing that the two proteins interact with each other (Fig. 2C). To identify what part of MCM7 was involved in the association with MCM-BP, in vitro translated Myc-tagged wild-type MCM7 and deletion mutants (Supplemental Fig. S4A) were incubated with

GST-MCM-BP or GST, and coprecipitates were analyzed by Western blotting. We found that the MCM7 deletion mutants, which lack the conserved MCM box that includes the canonical Walker A and B motifs, could not bind to MCM-BP (Fig. 2D, lanes 7,12). Consistent with this, MCM-BP could bind efficiently to a fragment of MCM7 that contained only this domain (Fig. 2D, lane 10). These experiments indicate that the interaction between MCM-BP and MCM7 relies on the MCM box of MCM7. We also found that a lysine-to-alanine point mutation within the conserved Walker A motif of MCM7 did not impair this interaction (Supplemental Fig. S4B). Given that the lysine residue in the Walker A motif is essential for nucleotide-binding and DNA-unwinding activities (You et al. 2002), this result suggests that ATP or its hydrolysis is not necessary for MCM7 to be associated with MCM-BP.

MCM-BP is imported in the nucleus in late S phase, when MCM2–7 dissociates from chromatin

The association of MCM-BP with MCM7, a subunit of the MCM2–7 helicase, led us to ask whether MCM-BP could play a role during DNA replication. Upon addition

of sperm nuclei to *Xenopus* interphase egg extracts, pre-RC formation on chromatin occurred within 10 min through binding of ORC2, MCM3, and MCM7 (Fig. 3A). Nuclear membrane formation around the chromatin occurred within 20 min (data not shown), allowing initiation of replication and binding of Replication Protein A (RPA) to chromatin (Fig. 3A,B), as expected. RPA, MCM3, and MCM7 were then released from chromatin during the ongoing DNA synthesis (Fig. 3A, right panel). MCM-BP was detected in the nucleus only by mid-S phase (Fig. 3A, left panel). However, despite its interaction with MCM7, MCM-BP was not stably bound to chromatin (Fig. 3A, right panel). Moreover, MCM-BP nuclear import strikingly correlated with MCM2–7 dissociation from chromatin and with the progressive increase of the MCM7–MCM-BP association (Fig. 3C). These data suggest that MCM-BP is involved in the final stages of DNA replication rather than during initiation of DNA synthesis or DNA elongation.

MCM-BP is involved in the dissociation of MCM2–7 at the end of S phase

To further define the role of MCM-BP during DNA replication, *Xenopus* interphase egg extracts were immunodepleted of MCM-BP using the affinity-purified polyclonal anti-MCM-BP antibody (Fig. 3D; Supplemental Fig. S5), and their replication profile was further analyzed. MCM-BP depletion did not significantly affect the total MCM2–7 content of the extract, in agreement with the large excess

of MCM2–7 complex in the extract (Fig. 3D). Recruitment of ORC2, MCM3, and MCM7 to chromatin was not significantly affected under these conditions (Fig. 3E). Similar experiments using membrane-free egg extracts—which assemble pre-RCs, including MCM2–7, but cannot activate DNA synthesis (Coue et al. 1998; Mimura and Takisawa 1998)—confirmed that MCM-BP depletion did not impair pre-RC assembly, judging from normal Orc2, Cdt1, and MCM2–7 loading onto chromatin (Supplemental Fig. S6). In complete extracts, loading onto chromatin of Cdc45 (a pre-IC component) (data not shown) and of PCNA (an elongation complex factor) (Fig. 3D) was also normal in MCM-BP-depleted extracts, and DNA replication was achieved with kinetics comparable with that of mock-depleted extracts (Fig. 3F). Conversely, under this condition, depletion of MCM-BP significantly affected MCM2–7 dissociation from chromatin during DNA synthesis (Fig. 3E; Supplemental Fig. S7A), in agreement with the finding that MCM-BP is imported into the nucleus only from mid-S phase (Fig. 3A). We found that the timing and dissociation of the MCM2–7 complex were reproducibly delayed when MCM-BP was depleted (Supplemental Fig. S7A), but not irreversibly blocked, probably due to

Figure 3. MCM-BP accumulates in the nucleus in late S phase and is not required for pre-RC formation and DNA replication, but is involved in MCM2–7 dissociation. (A) Time course of MCM-BP accumulation in the nucleus (left panel) and binding to chromatin (right panel). Sperm nuclei (3000 per microliter) were added to *Xenopus* interphase egg extracts, and then intact nuclei (lanes 3–9) and detergent-resistant chromatin fractions (lanes 12–18) were isolated at the indicated time points during DNA replication (see the Materials and Methods) and analyzed by Western blotting. A sample of egg extracts (0.5 μ L) (lanes 1,10) (LSS, low-speed supernatant) or insoluble material obtained by centrifugation of egg extracts (lanes 2,11) (no DNA) was also included as control. (B) Replication efficiency of the reaction described in A. (C) Time course of MCM-BP binding to MCM7. *Xenopus* sperm nuclei (XSP) were added to *Xenopus* interphase egg extracts, and intact nuclei were isolated and separated into chromatin and soluble nuclear fractions at different time points during DNA replication, as described in the Materials and Methods. (Top panel) MCM2–7 unloading from chromatin was assessed using anti-MCM3 and anti-MCM7 antibodies. (Bottom panels) Soluble nuclear fractions were immunoprecipitated with anti-MCM7 antibodies, and immunoprecipitates were analyzed by Western blotting with anti-MCM3, anti-MCM7, and anti-MCM-BP antibodies. (D) One microliter of mock- and MCM-BP-depleted (using the rabbit polyclonal anti-MCM-BP antibody) interphase egg extracts was analyzed by Western blotting with the indicated antibodies to confirm MCM-BP depletion. (E) MCM-BP regulates MCM2–7 unloading from chromatin during late S phase. *Xenopus* interphase egg extracts were immunodepleted with IgG fractions purified from preimmune serum (left panels) or with the rabbit polyclonal anti-MCM-BP antibody (right panels). Sperm nuclei (3000 per microliter) were added to *Xenopus* egg extracts, and chromatin fractions were isolated at the indicated times during DNA replication (see the Materials and Methods). Chromatin-bound proteins were analyzed by Western blotting using the indicated antibodies. (F) Replication efficiency of the reaction described in E.

alternative mechanisms that can clear the MCM2–7 after DNA replication (see the Discussion). We then investigated whether depletion of MCM-BP would inhibit replicon end-joining by analysis of replication products using alkaline gel electrophoresis. Replicating DNA isolated from MCM-BP-depleted extracts progressively accumulated high-molecular-weight DNA chains similarly to mock-depleted extracts, indicating that replicated DNA from adjacent replicons could converge normally and join in the absence of MCM-BP (Supplemental Fig. S7C). We conclude that MCM-BP depletion inhibits and delays MCM2–7 dissociation during S phase by a mechanism that does not involve replicon end-joining.

A similar inhibition of DNA synthesis-dependent removal of MCMs was observed in the presence of aphidicolin, an inhibitor of DNA polymerase α (Fig. 4A, cf. lanes 1 and 2). However, inhibition of MCM2–7 unloading by aphidicolin was accompanied by Chk1 phosphorylation (Fig. 4A, lanes 2,4) and accumulation of RPA onto chromatin (Zou and Elledge 2003; Byun et al. 2005; data not shown). Conversely, MCM-BP depletion did not induce checkpoint activation at 120 min after sperm addition, as judged by the lack of Chk1 phosphorylation and RPA accumulation (Fig. 4A, lane 3; see also Supplemental Fig. S8B; data not shown). Thus, the presence of chromatin-bound MCM2–7 alone is not sufficient to induce checkpoint response.

We conclude that DNA synthesis is necessary for MCM2–7 dissociation from chromatin, but that intra-S checkpoint activation is unlikely to be the mechanism whereby MCM dissociation is inhibited upon MCM-BP depletion. These data also confirm that MCM-BP is involved in a late step of DNA replication during MCM2–7 unloading from chromatin.

In order to substantiate the specificity of the effect observed in MCM-BP-depleted extracts, we tried to rescue MCM2–7 unloading in these extracts by adding 15 ng/ μ L recombinant MCM-BP, a concentration comparable with that of endogenous MCM-BP (10–20 ng/ μ L) (Fig. 4B). Recombinant MCM-BP clearly rescued MCM3 and MCM7 unloading in the late stage of S phase (Fig. 4C, cf. lanes 4 and 2; see also Supplemental Fig. S8A,C). In addition, MCM-BP released MCM2–7 from chromatin only at the end of S phase, but not during pre-RC or pre-IC formation. Indeed, incubation with recombinant MCM-BP had no effect on MCM2–7 in pre-RCs assembled in high-speed extracts (Fig. 4D), in which pre-RCs are efficiently formed but DNA replication cannot initiate, as described above.

We then investigated whether MCM-BP could accelerate MCM2–7 dissociation from chromatin and at what time during S phase. Chromatin was isolated at various times during S phase and was further incubated with recombinant MCM-BP. MCM proteins were efficiently dissociated from chromatin only when MCM-BP was added after 45 min (Fig. 4E), but not during pre-RC formation. In agreement with this result and with our observations using membrane-free egg extracts (Fig. 4D), recombinant MCM-BP could not release MCM2–7 from chromatin when initiation of DNA synthesis was blocked by p21 (Fig. 4E). Thus, p21 inhibits MCM2–7 removal, confirming that

Figure 4. Efficient MCM2–7 dissociation from chromatin involves MCM-BP and S-phase progression. (A) MCM-BP depletion does not cause checkpoint activation. Sperm nuclei (6000 per microliter) were added to mock- or MCM-BP-depleted (Δ BP) *Xenopus* interphase egg extracts in the absence or presence of 50 μ g/mL aphidicolin. Nuclei were isolated 120 min after sperm addition, and both chromatin-bound fractions (*top* panels) and soluble nuclear fractions (*bottom* panels) were analyzed by Western blotting using the indicated antibodies. (B) Purification of recombinant MCM-BP. His-tagged full-length MCM-BP was expressed and further purified by nickel chromatography. The purified protein was analyzed by SDS-PAGE followed by staining with Coomassie blue. (C) Purified recombinant MCM-BP rescues MCM3 unloading from chromatin in MCM-BP-depleted *Xenopus* egg extracts. Egg extracts were mock-depleted with IgG fractions purified from preimmune serum (lanes 1,3) or immunodepleted with the rabbit polyclonal anti-MCM-BP antibody (lanes 2,4). Then, buffer alone (lanes 1,2) or 0.2 μ M recombinant MCM-BP (rBP) (lanes 3,4) was added to the depleted extracts before sperm addition (3000 per microliter). One-hundred-twenty minutes later, chromatin fractions were isolated and analyzed by Western blotting for MCM3, ORC2, and H3. (D,E) Recombinant MCM-BP releases MCM2–7 from chromatin only in late S phase. Chromatin or nuclei were isolated from high-speed egg extracts (HSE) (D) or low-speed egg extracts (LSE) (E) treated with buffer or recombinant p21, and incubated with 3 μ M recombinant MCM-BP (rBP) in CPB buffer containing 0.2% Triton X-100. After 20 min of incubation at room temperature, soluble supernatants were removed by centrifugation, and chromatin fractions were analyzed by Western blotting using the indicated antibodies.

DNA synthesis is necessary for further MCM2–7 displacement from chromatin (Fig. 4E, cf. lanes 3,4 and 7,8) and recombinant MCM-BP cannot displace MCM2–7 under these conditions (Fig. 4E, cf. lanes 7 and 8). Similar results were obtained following aphidicolin treatment (data not shown).

Altogether, these results suggest that MCM-BP regulates the dissociation of MCM2–7 from chromatin after ongoing DNA synthesis, and that, during pre-RC or pre-IC assembly, MCM binding to chromatin is not affected.

We then wished to address the mechanism by which MCM2–7 is unloaded from chromatin by MCM-BP. Here we showed that MCM-BP binds to MCM7 but is not detected in the whole MCM2–7 complex. One explanation could be that, when MCM-BP binds to the MCM2–7 complex, the complex is simultaneously dissociated, resulting in its release from chromatin. To test this hypothesis, recombinant MCM-BP was added to *Xenopus* egg extracts and the composition of the MCM2–7 complex was analyzed by sucrose gradient density centrifugation. Recombinant MCM-BP induced a dramatic change in the sedimentation profile of the complex, as a large part of the MCM2–7 complex was dissociated in low-molecular-weight fractions (Fig. 5A). To identify the resulting complexes, pooled high- or low-molecular-weight fractions (H and L fractions in Fig. 5A) were immunoprecipitated with anti-MCM7 antibodies. High-molecular-weight fractions from control and extracts with excess MCM-BP (Fig. 5B, “H”) contained the MCM7 subunit but no MCM-BP (see also Fig. 2A). In low-molecular-weight fractions from control extracts (Fig. 5B, “L”), MCM7 was associated with MCM-BP and also with MCM3 and MCM4.

Conversely, in low-molecular-weight fractions from extracts with excess MCM-BP, only association of MCM7 with MCM-BP could be detected, but not with MCM2, MCM3, or MCM4 (Fig. 5B, cf. lanes 2 and 4). MCM3 immunoprecipitation showed that the association of MCM3 with MCM2 and MCM4 was also lost (Supplemental Fig. S9). These results imply that binding of MCM-BP to MCM7 not only releases MCM7 from the whole MCM2–7 complex, but also destabilizes the interactions of each individual subunit. We also noticed that endogenous MCM-BP, in the complex with MCM7, was replaced by recombinant MCM-BP under this condition, suggesting that the binding of MCM-BP to MCM7 is dynamic.

Then, we asked whether MCM-BP could directly dissociate the isolated MCM2–7 complex. To this aim, immunopurified MCM2–7 complex (see the Materials and Methods) was incubated with recombinant MCM-BP or Geminin, an inhibitor of MCM loading to chromatin, as a control. After incubation with MCM-BP, a substantial part of the MCM2–7 complex was dissociated, releasing MCM subunits in the eluate (Fig. 5C). Dissociation did not occur upon incubation with buffer alone, purified

Figure 5. Excess MCM-BP dissociates the MCM2–7 hexameric complex. (A) *Xenopus* interphase egg extracts were incubated or not with 2 μ M recombinant MCM-BP (rBP) for 1 h without sperm addition. They were then fractionated on a linear 7%–19% sucrose gradient. The indicated proteins were detected by Western blot analysis. (B) Pooled high-molecular-weight fractions (fractions 13–16; H) and low-molecular-weight fractions (fractions 23–26; L) were immunoprecipitated with anti-MCM7 antibodies (lanes 1–4) and analyzed by Western blotting. (C) The MCM2–7 complex was immunoprecipitated from *Xenopus* interphase egg extracts with anti-MCM7 antibodies. MCM7 precipitates were washed and incubated with purified recombinant MCM-BP (2 μ M) (lane 2) or Geminin (2 μ M) (lane 3) for 1 h. The proteins released in the eluate were analyzed by Western blotting with antibodies against different MCM proteins.

recombinant Geminin, or MCM2 (Fig. 5C; data not shown). Importantly, this reaction was not affected by either the absence of ATP or the presence of ATP analogs like ATP- γ S (Supplemental Fig. S10) and AMP-ANP (data not shown). From this set of experiments, we conclude that MCM-BP can dissociate the MCM2-7 complex in a reaction that does not require ATP.

Finally, we investigated the consequences of silencing *MCM-BP* in HeLa cells by using siRNAs. In HeLa cells, most of MCM-BP was observed only in the chromatin-free fraction supernatants after cell fractionation (Fig. 6A; Materials and Methods) and did not cosediment with the MCM2-7 complex in glycerol density gradient fractionation (Fig. 6B) like in *Xenopus* egg extracts. siRNA efficiently depleted MCM-BP from HeLa cells (Fig. 6A, cf. lanes 1 and 2), and we examined DNA replication by monitoring incorporation of BrdU into the cells. In comparison with

cells transfected with control siRNA, cells transfected with *MCM-BP* siRNA showed a slight increase in the number of cells in S and G2/M phase of the cell cycle but similar BrdU incorporation (Fig. 6C,D). To directly assess the effect of *MCM-BP* silencing on cell cycle progression, cells were synchronized at the G1/S boundary by double-thymidine block after two rounds of siRNA transfection. Cells were then released from the block, and cell lysates were prepared at different time points and analyzed by immunoblotting. Concomitantly, we evaluated cell cycle progression by FACS analysis. More than 80% MCM-BP was depleted after two rounds of transfection with siRNA SMART pools specific for *MCM-BP* (Fig. 7A). FACS analysis again showed that S-phase progression was not significantly affected by MCM-BP depletion (Fig. 7B). However, consistent with the *Xenopus* data, dissociation of MCM7 from chromatin fractions was delayed compared with control cells. We also observed that entry into mitosis occurred more slowly in the absence of MCM-BP, as judged by the delayed accumulation of phosphorylation of H3 at Ser 10 (Fig. 7A) in comparison with control cells. Altogether, our data suggest that, in human cells, MCM-BP regulates proper G2/M progression by controlling MCM2-7 dissociation from chromatin rather than being involved in bulk DNA replication.

Discussion

Clearing the replication complex from chromatin is essential before mitosis, and several mechanisms are involved in this function. The findings described in this study suggest that MCM-BP contributes specifically to the dissociation of the MCM2-7 ring complex.

MCM-BP is highly conserved among higher eukaryotes from fission yeast to mammals, but we were unable to detect a MCM-BP ortholog in *Saccharomyces cerevisiae*. However, in this organism, MCM2-7 is inactivated by nuclear export in a cdk-dependent manner (Nguyen et al. 2001). In other organisms, including fission yeast, MCM2-7 is constitutively localized in the nucleus (Yanow et al. 2001), and therefore additional mechanisms to inhibit MCM2-7 would be required. In fission yeast, global analyses showed that the MCM-BP homolog localizes in the nucleus and appears essential for viability (Matsuyama et al. 2006; Kim et al. 2010), suggesting a crucial role during the cell cycle. This hypothesis is supported by the finding that plant cells with a MCM-BP mutant have a defect in G2/M cell cycle progression. Our results show that, in *Xenopus* egg extracts, MCM-BP is involved in the release of the MCM2-7 complex from chromosomes during the late stage of S phase, in agreement with the observations in other organisms. Moreover, in human cells, where the first MCM-BP gene was identified, we detected very little association of MCM-BP with chromatin, and *MCM-BP* silencing induced delayed MCM7 dissociation from chromatin. Although in our experiments we used detergent concentrations that, in the *Xenopus* system, are known to entirely remove the nuclear membrane but leave on chromatin all the proteins involved in DNA replication, we cannot rule out the possibility that a minor fraction of MCM-BP might

Figure 6. *MCM-BP* silencing in HeLa cells increases the G2/M population. (A) Human HeLa cells were transfected with *Luciferase* siRNA (lanes 1,3,5) or *MCM-BP* siRNA (lanes 2,4,6). Cells were fractionated (see the Materials and Methods) and immunoblotted to assess the expression of MCM-BP, Actin, MCM2, and H3. (B) After CSK extraction, soluble HeLa supernatant was loaded onto a linear 5%–25% glycerol gradient and centrifuged at 26,000 rpm in an SW55Ti rotor for 22 h. Fractions were subjected to SDS-PAGE, and the indicated proteins were detected by Western blot analysis. (C,D) Forty-eight hours after two rounds of transfection with *Luciferase* or *MCM-BP* siRNAs, HeLa cells were incubated or not with BrdU for 15 min and stained with propidium iodide, and their DNA content was analyzed by flow cytometry. (C) The DNA content is shown on the X-axis, and the relative number of cells is indicated on the Y-axis. (D) The intensity of BrdU incorporation is indicated on the logarithmic Y-axis; the DNA content is shown on the X-axis.

Figure 7. *MCM-BP* silencing in HeLa cells results in defective mitotic entry. HeLa cells were synchronized by double-thymidine block and release. Cells transfected with *Luciferase* or *MCM-BP* siRNAs were incubated with 2 mM thymidine for 15 h followed by washing with prewarmed PBS and medium. Cells were released into fresh medium for 9 h and then blocked again with 2 mM thymidine for 17 h. Cells were washed with prewarmed PBS and medium before release into fresh medium. Cells were processed for immunoblotting (A) and FACS analysis (B) at the indicated time points after synchronization.

bind to chromatin in human cells in less stringent conditions. Interestingly, Sakwe et al. (2007) showed that MCM-BP stayed longer on chromatin in late S to G2 phase in human cells, when MCM2-7 was almost displaced from DNA, in agreement with the idea that MCM-BP functions as an unloader of MCM2-7. As reported previously in human cells, we found that *MCM-BP*-silenced cells continued to grow and incorporate BrdU, suggesting that the defect in these cells was not directly due to impaired DNA replication.

MCM-BP and the regulation of unloading of the MCM2-7 ring complex

Dissociation of MCM2-7 from chromatin at the end of S phase is important to prevent rereplication of DNA and to ensure genome duplication only once per cell cycle. In this study, we provide evidence that MCM-BP is involved in DNA replication-dependent MCM2-7 unloading from chromatin in *Xenopus* interphase egg extracts. After loading onto chromatin during pre-RC formation, MCM2-7 tightly associates with chromatin, and progression of DNA replication is necessary for its dissociation. We also demonstrate that MCM-BP regulates the dissociation of the MCM2-7 complex by physically interacting mainly with its MCM7 subunit. Our results suggest that MCM-BP is transiently recruited to chromatin to release MCM2-7 from DNA at the end of the synthesis of replicons. This is in agreement with the observation that MCM-BP enters the nucleus during S-phase progression, and that its nuclear import kinetics correlates with MCM2-7 release from chromatin. In *Xenopus*, replicons are short, with DNA replication origins spaced every 10–20 kb (Hyrien and Mechali 1993;

Walter and Newport 1997). The pre-RC formation extends for ~20 min, after which the replication forks proceed at a speed estimated to be 0.6 kb/min (Blow et al. 2001; Lemaitre et al. 2005). The first replicons to be activated will therefore reach their termination point after 35–45 min. This is the time when MCM-BP is imported into the nucleus and the MCM2-7 complex starts to be released from chromatin. Since MCM-BP binding to the MCM2-7 complex simultaneously promotes its dissociation from chromatin, chromatin-bound MCM-BP is unlikely to be detected during this reaction. A possible dynamic interaction of MCM-BP with MCM7 could also be part of a resetting mechanism, which would allow the unloaded MCM2-7 to be used in subsequent cell cycles.

Several observations, including those here, indicate that DNA replication has to be induced to allow MCM2-7 dissociation from chromatin. MCM2-7 dissociation is abolished when initiation of DNA synthesis is inhibited in aphidicolin-treated extracts, despite the presence of nuclear MCM-BP. Similarly, recombinant MCM-BP cannot dissociate MCM2-7 from chromatin that was isolated early in S phase, including licensed chromatin in membrane-depleted high-speed egg extracts (Fig. 4D,E). We think that the stable association of MCM2-7 with the pre-RC or pre-IC complex might prevent MCM-BP from functioning at this stage. MCM2-7 dissociation by MCM-BP might therefore require the MCM2-7 helicase to have left the replication origins and traveled along the DNA. An additional possibility could be that MCM-BP is required for inactivation of the MCM2-7 complex in the nucleoplasm to prevent its reassociation with chromatin at the end of S phase.

Altogether, our data suggest that MCM-BP controls the opening of the MCM2-7 complex at the end of S phase through its interaction with MCM7. Interestingly, the MCM7/4 site is the active site involved in DNA unwinding by the MCM2-7 complex (Kanter et al. 2008; Bochman and Schwacha 2009), suggesting that disruption of this site will not only dissociate the MCM complex, but also inactivate it.

Implications of MCM2-7 clearing from chromatin at the end of S phase

The release of the MCM2-7 complex from chromatin might be crucial at three points: twice during S phase (at the end of replication of replicons and to remove the MCM complexes loaded at silenced origins) and once at mitosis. Here we show that MCM-BP depletion does not alter DNA synthesis or affect Chk1 phosphorylation, suggesting that loss of MCM-BP is not sufficient for inducing rereplication. In metazoans, Geminin synthesis and Cdt1 degradation are independent and redundant mechanisms to prevent rereplication. We cannot exclude the possibility that MCM-BP-dependent removal of MCM2-7 from chromatin provides additional safeguards against rereplication.

Before chromosome segregation in mitosis, DNA-bound components during S phase might become obstacles to the formation of metaphase chromosomes and/or chromosome segregation (Yanagida 2009). Therefore, these components

might have to be removed. Failure to do so may result in defects in the mitotic chromosomes' assembly (Cuvier et al. 2006, 2008). The MCM2–7 complex tightly binds in excess to interphase chromatin (resistant to high-salt treatment), and its removal from chromatin is therefore critical for progression into mitosis. We found that silencing of *MCM-BP* in human cells also resulted in defects in mitotic entry (Fig. 7) similar to what was observed in plant cells (Takahashi et al. 2010). In addition to the role of mitotic cyclin-dependent kinases in cleaning replicated chromosomes for mitosis, MCM-BP also could take part in opening and dissociating the MCM2–7 ring complex from chromatin. MCM-BP absence is not sufficient to completely block MCM2–7 dissociation from chromatin, and cells may have evolved different but redundant mechanisms to ensure the removal of pre-RC. At least two mechanisms might contribute to the complete dissociation of MCM2–7 from replicated chromatin. First, a high concentration of recombinant CDK2–Cyclin E negatively regulates pre-RC assembly (Hua et al. 1997) and accelerates the interphase/mitosis transition (Moore et al. 2002), while a comparable concentration of S-CDK in nucleoplasmic extracts does not block Cdt1-dependent MCM2–7 loading and DNA replication (Arias and Walter 2005). Enrichment of Cdk2–Cyclin E in the nucleus may contribute to some late processes in the cell cycle, including MCM2–7 dissociation. Second, the Topoisomerase II-dependent pathway, which is responsible for removing RPA and ORC reservoirs from chromatin (Cuvier et al. 2008), could also play a role in MCM2–7 clearing after replication. Indeed, we observed that ICRF-193, an inhibitor of Topoisomerase II, could also suppress the dissociation of MCM2–7 in late S phase (data not shown). These mechanisms would ensure that the removal of pre-RC occurs before mitosis, thus eliminating the obstacles for mitotic chromosome assembly.

Materials and methods

Xenopus oocytes, eggs, and egg extracts

Interphasic low-speed supernatants and high-speed supernatants were prepared as described (Maiorano et al. 2005; Lutzmann and Mechali 2008). Low-speed supernatants were finally clarified by centrifugation at 20,000 rpm in an SW55Ti rotor for 20 min. Western blot analyses of oocytes were performed as described (Lemaître et al. 2002).

Plasmids and proteins

Full-length *Xenopus* MCM-BP and MCM7 cDNAs were purchased from Imagen. *Xenopus* MCM2 cDNA was provided by D. Maiorano. Full-length MCM-BP and MCM2 cDNA and the cDNA fragment of MCM7 that encodes amino acids 545–720 were cloned into pET16b to be expressed as His-tagged proteins. Full-length MCM-BP was also subcloned in the pET24-GST-TEV vector (GST-tagged MCM-BP). His- and GST-tagged fusion proteins were expressed in *Escherichia coli* BL21 codon plus RIL cells (Stratagene) and were purified as described (Lutzmann et al. 2006). Purified proteins were loaded onto PD-10 desalting columns equilibrated with XB buffer (10 mM HEPES/KOH at pH 7.7, 100 mM KCl, 0.1 mM CaCl₂, 1 mM MgCl₂, 2% sucrose)

containing 1 mM DTT, and then concentrated with Vivaspin (Millipore). His-tagged Geminin was purified as described (Lutzmann et al. 2006). Translation in reticulocyte lysates was performed according to the manufacturer's instructions (TNT Reticulocyte Lysates System, Promega). For translation, MCM-BP and MCM7 cDNAs were cloned into the pcDNA3 vector. The MCM7 mutant in which Lys 386 was substituted by alanine was generated by PCR-mediated mutagenesis.

Antibodies

Anti-*Xenopus* MCM-BP antisera were obtained by immunizing rabbits with recombinant, full-length His10-tagged MCM-BP; expressed in *E. coli*; and purified by SDS-PAGE. Antisera were further affinity-purified with the recombinant protein immobilized onto a nitrocellulose membrane. Anti-*Xenopus* MCM7 antibodies were raised as described above, by immunizing rabbits with a recombinant His10-tagged MCM7 fragment (545–720). Anti-human MCM2 and MCM5 rabbit antibodies that cross-react with *Xenopus* MCM2 and MCM5 and anti-human MCM7 mouse monoclonal antibody that also recognizes *Xenopus* MCM7 were purchased from Abcam. A mouse monoclonal antibody against PCNA and a polyclonal anti-histone H3 antibody were from Sigma and Abcam, respectively. The anti-Phospho-Chk1 and anti-Phospho-Myc antibodies were from Cell Signaling. The antibody against *Xenopus* Cdc45 was kindly provided by J. Walter. Antibodies against *Xenopus* Orc2, MCM3, Cdt1, and RPA, and human MCM-BP were described previously (Maiorano et al. 2005; Sakwe et al. 2007; Lutzmann and Mechali 2008).

Nuclei and chromatin isolation from *Xenopus* egg extracts

Nuclei and chromatin fractions were prepared from *Xenopus* egg extracts incubated with 3000–6000 demembrated sperm nuclei per microliter. At the end of the incubation period, the reaction was diluted with 4 vol of CPB (50 mM KCl, 5 mM MgCl₂, 20 mM HEPES/KOH at pH 7.7, 2% sucrose), and nuclei were purified by centrifugation at 6000g through a sucrose cushion containing 0.7 M sucrose dissolved in CPB for 5 min. For chromatin purification, nuclei pellets were solubilized in 40 μ L of CPB containing 0.1% NP-40. Chromatin fractions were collected by centrifugation at 10,000g for 5 min. The final supernatant was used as nuclear lysates for immunoprecipitations that were performed with antibodies bound to protein A-agarose (Roche).

Immunoprecipitations

Five micrograms of the antibody for immunoprecipitation was incubated with protein A-agarose beads for 2 h at room temperature and washed in XB buffer. *Xenopus* egg extracts were fivefold diluted in XB and incubated with the antibody-bound beads for 1 h at 4°C. Beads were then washed with XB twice, with XB containing 0.1% Triton X-100 once, and with XB alone twice. Proteins were finally eluted with SDS sample buffer and analyzed by SDS-PAGE.

Sucrose and glycerol gradients

Sucrose or glycerol density gradient centrifugation was performed as described (Lutzmann and Mechali 2008). Briefly, egg extracts were diluted fivefold in XB and centrifuged at 12,000g for 10 min at 4°C. Cleared egg extract or human cell lysate (200 μ L) was layered onto a linear gradient prepared in XB supplemented with protease inhibitors. The gradient was then centrifuged at 26,000g for 22 h at 4°C in a SW 55Ti rotor, and fractions were collected.

In vitro dissociation of the MCM2–7 complex

To follow the disassembly of MCM2–7 complex by MCM-BP, *Xenopus* interphase egg extracts were incubated with 2 μ M recombinant MCM-BP or Geminin for 30 min at room temperature. Egg extracts were cleared by centrifugation and analyzed by sucrose density gradient centrifugation as described above. When sperm chromatin was added to extract, dilution of extracts was performed in XB containing 0.2% Triton X-100 and centrifuged to remove insoluble materials from egg extracts. For immunopurification of the MCM2–7 complex, purified rabbit polyclonal anti-MCM7 antibodies or control IgGs were coupled to protein A-agarose beads, incubated with diluted *Xenopus* egg extracts for 1 h at 4°C, and then washed as described above. Proteins specifically bound to the beads were incubated with 2 μ M MCM-BP or Geminin in 50 μ L of XB supplemented with an ATP-regenerating system (10 mM creatine phosphate, 10 μ g/mL creatine kinase, 1 mM ATP, 1 mM $MgCl_2$) for 30 min at room temperature. To see the effect of recombinant MCM-BP to the MCM2–7 on chromatin, intact nuclei were isolated from egg extracts as described above. Then, nuclei pellets were incubated for 15 min at room temperature in 40 μ L of CPB containing 0.2% Triton X-100 and an ATP-regenerating system with or without recombinant MCM-BP at a concentration of 3 μ M. Chromatin fractions were collected by centrifugation at 10,000g for 5 min and washed once in XB. Chromatin-bound proteins were analyzed by SDS-PAGE and Western blotting.

Human tissue culture cells

Asynchronous HeLa cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. SMART pool siRNAs specific for MCM-BP (J-014474-09, J-014474-10, and J-014474-11) and control siRNA (SMART pool nontargeting pool) were obtained from DharmaFect. The siRNAs (33 nM) were transfected into HeLa cells with Lipofectamine2000 according to the manufacturer's instructions (Invitrogen). Cells were subcultured for 48 h after two rounds of transfection with siRNA. Subsequently, cells were labeled with 10 μ g/mL BrdU for 15 min. Cells were fixed with 70% ethanol, incubated with 20 μ g/mL RNase A for 1 h, and treated with 2 N HCl for 30 min. Cells were incubated with anti-BrdU antibodies for 1 h and then stained with fluorescent-conjugated secondary antibodies and propidium iodide according to standard procedures. FACS analysis was performed with a FACSCalibur flow cytometer (BD Biosciences).

Isolation of chromatin fractions from synchronized human cells

HeLa cells were synchronized at the G1/S boundary by double-thymidine block with 2 mM thymidine. Cells were harvested, suspended in CSK buffer (10 mM PIPES-KOH at pH 6.8, 100 mM NaCl, 300 mM sucrose, 1.5 mM $MgCl_2$, 0.1% Triton X-100) containing 5 mM EDTA, 1 mM DTT, 0.1 mM sodium vanadate, 1 mM NaF, 10 mM β -glycerophosphate, 0.1 μ M tautomycin, and 0.5 mM PMSF; and lysed for 10 min on ice. Chromatin-enriched fractions were collected by centrifugation at 1300g for 3 min and washed in CSK buffer. For fractionation by sucrose or glycerol density gradient, soluble supernatants were further clarified by centrifugation at 12,000g for 10 min.

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MCM-BP regulates unloading of the MCM2–7 helicase in late S phase

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